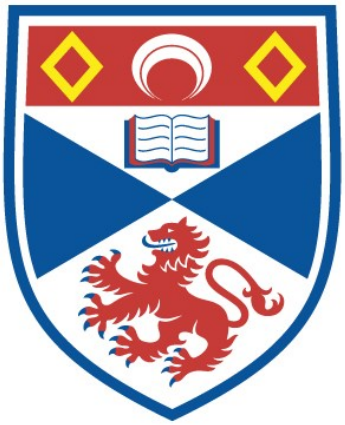
NEUROTROPHIC ACTIONS OF GDNF AND NEURTURIN IN THE DEVELOPING AVIAN NERVOUS SYSTEM AND CLONING AND EXPRESSION OF THEIR RECEPTORS

Anna Buj-Bello

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



1997

NEUROTROPHIC ACTIONS OF GDNF AND NEURTURIN IN THE DEVELOPING AVIAN NERVOUS SYSTEM AND CLONING AND EXPRESSION OF THEIR RECEPTORS

A thesis submitted to the University of St. Andrews
for the degree of
Doctor of Philosophy (Ph.D.)
by
Anna Buj-Bello
September, 1997

School of Biomedical Sciences
Bute Medical Buildings
St.Andrews

"Description is easy and infinite, explanation is difficult and limited"

Hippocrates

I dedicate this thesis to Esber and to my parents,
for their love and patience

II. ABSTRACT

The main aim of this project was to determine the neurotrophic actions of glial cell line-derived neurotrophic factor (GDNF) and neurturin, two novel members of the transforming growth factor-beta superfamily of proteins, on neurons from the peripheral nervous system and to identify their receptors. It is found that GDNF promotes the survival of multiple populations of chicken sensory and autonomic neurons in culture throughout development. Whereas sympathetic, parasympathetic and propioceptive neurons become less responsive to GDNF with age, enteroceptive and sensory cutaneous neurons become more responsive to GDNF. GDNF mRNA is expressed in the tissues innervated by these neurons, and developmental changes in its expression in several tissues mirror the changing responses of the innervating neurons to GDNF. These results have changed the previous notion that GDNF is a highly specific neurotrophic factor for motoneurons and dopaminergic neurons. It is shown that neurturin, which is structurally related to GDNF, also promotes the *in vitro* survival of embryonic chicken sensory and autonomic neurons. Thus, GDNF and neurturin compose a novel subfamily of homologous neurotrophic factors with a similar pattern of activity.

The cloning of chicken GDNF receptor-alpha (GDNFR-α) and a novel receptor termed neurturin receptor-alpha (NTNR-α) is reported. GDNFR-α and NTNR-α are homologous receptors linked to the membrane via a glycosyl-phosphatidylinositol linkage. It is shown that ectopic co-expression in neurons of GDNFR-α with RET (rearranged during transfection), a transmembrane receptor tyrosine kinase, confers a survival response to GDNF, but not neurturin, and that co-expression of NTNR-α with RET confers a survival response to neurturin, but not GDNF. GDNFR-α and NTNR-α mRNAs are widely expressed in the nervous system, including GDNF and neurturin responsive neurons, and in non-neuronal tissues. These findings indicate that GDNF and neurturin promote neuronal survival by signalling via similar multicomponent receptors that consist of a common transducing receptor tyrosine kinase and a member of a newly emerging family of glycosyl-phosphatidylinositol-linked receptors that confer ligand-specificity.

III. DECLARATIONS

I, Anna Buj-Bello, hereby certify that this thesis, which is approximately 53,000 words in length, has been written by me, that is the record of work carried out by me and that it has not been submitted in any previous application for a higher degree.

Date 21st. July 1997 Signature of candidate

I was admitted as a research student in October, 1994 and as a candidate for the degree of PhD in October 1994; the higher study for which this is a record was carried out in the University of St.Andrews between 1994 and 1997.

Date 21st. July 1997 Signature of candidate

I hereby certify that the candidate has fulfilled the conditions of the Resolution and Regulations appropiate for the degree of Ph.D. in the University of St. Andrews and that the candidate is qualified to submit this thesis in application of that degree.

Date 31/7/97

Signature of supervisor

IV. COPYRIGHT

In submitting this thesis to the University of St.Andrews I understand that I am giving permission for it to be made available for use in accordance with the regulations of the University Library for the time being in force, subject to any copyright vested in the work not being affected thereby. I also understand that the title and abstract will be published, and that a copy of the work may be made and supplied to any bona fide library or research worker.

Date 21st. July 1997 Signature of candidate

V. CONTENTS

VII. ABBREVIATIONS

Aids acquired immuno-deficiency syndrome

AIF apoptosis-inducing factor

BDNF brain-derived neurotrophic factor

bFGF basic fibroblast growth factor

BH bcl-2 homology region

BMP bone morphogenetic protein

bp base pair

BSA bovine serum albumin

°C Celsius degree

CDF cholinergic differentiation factor

cDNA complementary deoxyribonucleic acid

ChAT choline acetyl-transferase

Cip calf intestine phosphatase

cm centimetre

CNS central nervous system

CNTF ciliary neurotrophic factor

CNTFRα CNTF receptor alpha

CT-1 cardiotrophin-1

ddATP dideoxyadenosine triphosphate

ddCTP dideoxycytidine triphosphate

ddGTP dideoxyguanosine triphosphate

ddNTP dideoxynucleotide triphosphate

ddTTP dideoxythymidine triphosphate

DIG digoxigenin

DMTG dorsomedial trigeminal ganglion

DN deep nucleus

DNA deoxyribonucleic acid

DNase deoxyribonuclease

dNTP deoxynucleotide triphosphate

DPP decapentaplegic

DRG dorsal root ganglion

DTT dithiothreitol

DVR decapentaplegic-Vg1-related

E embryonic day

EC_{50} half-maximally effective concentration

E. coli Escherichia coli

EDN endothelin

EDNR endothelin receptor

EDTA ethylenediaminetetraacetic acid

ENS enteric nervous system

FGF fibroblast growth factor

FMTC familiar medullary thyroid carcinoma

FSH follicle-stimulating hormone

F12 Ham nutrient mixture F-12

F14 Ham nutrient mixture F-14

g gramme

GAPDH glyceraldehyde-3-phosphate dehydrogenase

GC granule cell

G-CSF granulocyte-colony-stimulating factor

GDF growth factor differentiation

GDNF glial cell line-derived neurotrophic factor

GDNFR-α GDNF receptor alpha

GPA growth-promoting activity

GPI glycosyl-phosphatidylinositol

h hour

HBSS Hank's balanced salt solution

HIHS heat inactivated horse serum

ICE interleukin-1ß-converting enzyme

i.e. id est, that is

IGF insulin growth factor

IL-6 interleukin-6

IL-6-Rα IL-6 receptor alpha

kb kilobase

k_d dissociation constant

kDa kilodalton

L litre

LB Luria broth

LIF leukaemia inhibitory factor

LIFRß LIF receptor beta

L15 Leibovitz's L15 nutrient mixture

M molar

mA milliamper

MAD mother against DPP

MAPK mitogen-activated protein kinase

mCi millicurie

MEN multiple endocrine neoplasia

µg microgram

µl microlitre

µm micrometre

mg milligram

min. minute

MIS Müllerian inhibiting substance

ml millilitre

mm millimetre

mM millimolar

MOPS 4-Morpholinepropanesulfonic acid

MPP+ methyl-phenyl-pyridine

MPTP 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine

mRNA messenger ribonucleic acid

NC neural crest

ng nanogram

NGF nerve growth factor

NT neurotrophin

NTN neurturin

NTNR-α NTN receptor alpha

OD optical density

6-OHDA 6-hydroxydopamine

OP osteogenic protein

ORF open reading frame

OSM oncostatin M

O-2A oligodendrocyte-type-2-astrocyte

P postnatal

pBS Bluescript plasmid

PBS phosphate buffered saline

PC Purkinje cell

PCR polymerase chain reaction

PD Parkinson disease

PDGF platelet-derived growth factor

PEG polyethylene glycol

pfu unit forming plaque

pg picogram

PIPLC phosphatidylinositol phospholipase C

pmn progressive motor neuropathy

PNS peripheral nervous system

P-ORN poly-DL-ornithine

PTC papillary thyroid carcinoma

RET rearranged during transfection

RH random hexanucleotides

RNase ribonuclease

rpm revolutions per minute

RT reverse transcription

RT room temperature

RTK receptor tyrosine kinase

RT-PCR reverse transcription-polymerase chain reaction

SCG superior cervical ganglion

SDS sodium dodecyl sulphate

SH2 Src homology 2

SM sodium-magnesium

STE sodium-tris-EDTA

STET sucrose-Triton-EDTA-tris

STK serine/threonine kinase

TAE tris-acetate-EDTA

TBE tris-boric acid-EDTA

TßR TGF-ß receptor

TGF-ß transforming growth factor beta

TNF tumor necrosis factor

TNFR TNF receptor

TMN trigeminal mesencephalic nucleus

tRNA transfer ribonucleic acid

TrnR TGF-ß-related neurotrophic factor receptor

UV ultraviolet

VLTG ventrolateral trigeminal ganglion

vol. volume

VRC vanadyl-ribonucleoside complex

$^{V}/_{V}$ volume/volume

W Watts

VII. LIST OF FIGURES

CHAPTER 2

2.1. Location of the trigeminal and nodose ganglia, and the trigeminal mesencephalic nucleus in the chicken embryo.

2.2. Survival responses of E6, E8, E10 and E12 ciliary neurons to GDNF.

2.2.(A). Bar chart comparing the percent survival of ciliary neurons in control cultures and cultures containing GDNF, CNTF, and GDNF plus CNTF.

2.2.(B). Graph of the dose-responses of ciliary neurons to GDNF.

2.3. Survival responses of E8, E10, E12 and E14 sympathetic neurons to GDNF.

2.3.(A). Bar chart comparing the percent survival of sympathetic neurons after 48 hours incubation in control cultures and cultures containing GDNF, NGF, and GDNF plus NGF.

2.3.(B). Graph of the dose-responses of sympathetic neurons to GDNF.

2.4. Survival responses of TMN neurons to GDNF.

2.4.(A). Bar chart of the percent survival of E8, E10 and E12 TMN neurons after 48 hours incubation in control cultures and cultures containing GDNF, BDNF, and GDNF plus BDNF.

2.4.(B). Graph of the dose-responses of E10 TMN neurons to GDNF and BDNF.

2.5. Survival responses of E6, E8, E10 and E12 nodose neurons to GDNF.

2.5.(A). Bar chart comparing the percent survival of nodose neurons after 48 hours incubation in control cultures and cultures containing GDNF, BDNF, and GDNF plus BDNF.

2.5.(B). Graph of the dose-responses of nodose neurons to GDNF.

2.6. Survival responses of E6, E8, E10 and E12 DMTG and VLTG neurons to GDNF.

2.6.(A). Bar chart of the percent survival of DMTG neurons after 48 hours incubation in control cultures and cultures containing GDNF, NGF, and GDNF plus NGF.

2.6.(B). Bar chart of the percent survival of VLTG neurons after 48 hours

incubation in control cultures and cultures containing GDNF, BDNF, and GDNF plus BDNF.

2.7. TMN neurons cultured in the presence of GDNF and BDNF.

2.7.(A). Photomicrograph of E12 TMN neurons after 48 h of incubation in cultures containing 10 ng/ml of GDNF.

2.7.(B). Photomicrograph of E12 TMN neurons after 48 h of incubation in cultures containing 10 ng/ml of BDNF.

2.8.(A). Expression of GDNF mRNA in chicken and rat tissues.

2.8.(B). Developmental changes in the expression of GDNF mRNA in tissues innervated by GDNF-responsive neurons.

CHAPTER 3

3.1. Bar chart comparing the percent survival of E10 chicken ciliary ganglion neurons grown with neurturin, GDNF and neurturin plus GDNF for 48h.

3.2. Bar chart of the percent survival of E14 chicken DMTG neurons after 48h of incubation in cultures containing neurturin, GDNF, and neurturin plus GDNF.

3.3. Bar chart showing the survival of E10 chicken lumbar chain sympathetic neurons in culture after 48h of incubation in the presence of neurturin, GDNF, and neurturin plus GDNF.

3.4. Bar chart of E10 chicken nodose ganglion neurons cultures grown for 48h with neurturin, GDNF and neurturin plus GDNF.

CHAPTER 4

4.1. Nucleotide sequence of the chicken GDNFR-α cDNA corresponding to the 5'->3' strand.

4.2. Nucleotide sequence of the chicken NTNR-α cDNA corresponding to the 5'->3' strand.

4.3. Aligned protein sequences of rat and chicken GDNFR-α.

4.4. Aligned protein sequences of chicken GDNFR-α and NTNR-α.

4.5. Effect of PIPLC treatment on E6 chicken nodose ganglion neurons cultured in

the presence of either GDNF or BDNF.

4.6. Effect of PIPLC treatment on E6 chicken nodose ganglion neurons cultured in the presence of either neurturin or BDNF.

4.7. Effect of PIPLC treatment on E14 chicken sympathetic lumbar chain neurons cultured in the presence of either GDNF or NGF.

4.8. Bar chart of the percent survival of P4 mouse SCG neurons 48 hours after microinjection with expression plasmids for GDNFR-α, NTNR-α and RET both singularly and in combination.

4.9. Bar chart of the percent survival of P4 mouse SCG neurons 48 hours after microinjection with expression plasmids for GDNFR-α, NTNR-α and RET both singularly and in combination, in the presence of 50 ng/ml of either neurturin or GDNF.

4.10. Expression of GDNFR-α and NTNR-α mRNAs in the chicken embryo at E10.

4.11. Expression of GDNFR-α and NTNR-α mRNAs in the chicken embryo at E18.

4.12. Developmental changes in the expression of GDNFR-α and NTNR-α mRNAs in the chicken embryo.

4.13. Transverse sections of the lumbar region of E10 and E18 chicken embryos hybridized with DIG-labelled GDNFR-α and NTNR-α antisense riboprobes.

4.14. Sagital section of the cerebellum of E18 chicken embryo hybridized with DIG-labelled GDNFR-α antisense riboprobe.

4.15. RT-PCR detection of endogenous GDNFR-α and NTNR-α transcripts in chicken ciliary, nodose, dorsomedial trigeminal ganglion, and sympathetic lumbar chain neurons.

VIII. ACKNOWLEDGEMENTS

I am extremely grateful to:

Professor Alun M. Davies for giving me the opportunity of doing research in his laboratory and for his supervision since 1991, especially during this project.

Dr. Vladimir L. Buchman for his teaching, supervision and help in my work in Molecular Biology.

Dr. Joan X. Comella for guiding and supporting me in research since 1990.

Dr. Sean L. Wyatt for reading and commenting on this thesis.

Dr. Jimi Adu, Dr. Luzia G.P. Piñón, Dr. Miguel Chinchetru and Jane V. Thompson for collaboration in chapter 4.

Antony R. Horton for collaboration in chapters 2 and 4.

Epaminondas Doxakis for assistance with the RT-PCR technique.

Dr. Natalia Ninkina for advice during this project.

Thanks, for your help and friendship.

Dr. Arnon Rosenthal for supplying rodent GDNF and GDNFR-α cDNAs, RET cDNA and PIPLC, and for helpful comments.

Finally, I would like to thank Action Research for funding this project.

VIII. CHAPTER 1

GENERAL INTRODUCTION

1. GENERALITIES

The nervous system is the most complex organ structure in the human body. Despite the relative simplicity of its basic units, neurons and glial cells, the complexity of the nervous system is due to (1) the large number of neurons, there are about 10^{11} neurons in the adult human brain, (2) the large number of different kinds of nerve cells, probably about 10,000 different types, (3) the large number of connections that a neuron receives and establishes, i.e., the cerebellar Purkinje cell receives aproximately 150,000 contacts, and (4) the precise patterns of connections between neurons and with cells from other tissues. It is considered that the different functions of the mature human nervous system -like perception, motor coordination, memory, language, affection, conciousness, thought- depend on the establishment and consolidation of specific patterns of neuronal connections. A first step to get insights into this functional network is to understand the development of the nervous system.

1.1. DEVELOPMENT OF THE VERTEBRATE NERVOUS SYSTEM

During the early development of the vertebrate embryo, the monolayered blastula is transformed into a three-layered structure through a process of morphogenetic movements termed gastrulation. These three layers are: the endoderm, a tube that constitutes the primitive gut, is the innermost layer; the ectoderm, the epithelium that covers the embryo, is the outermost layer; and the mesoderm, between the two, is a layer of primary and secondary mesenchyme cells. Later, during organogenesis, the various organs of the body are formed. The primitive gut gives rise to the epithelial components of the pharynx, esophagus, stomach, intestine, and the associated structures like, the salivary glands, liver, pancreas, trachea, and lungs. The primitive mesenchyme gives rise to connective tissues (cartilage, bone, fibrous tissue), muscles, and the vascular and urogenital

systems. Finally, the ectoderm gives rise to the epidermis and the nervous system.

The development of the vertebrate nervous system is initiated during gastrulation by a process termed neural induction. The ectoderm close to the midline is induced to become neural ectoderm by the underlying notochord and mesoderm adjacent to it. During a post-gastrulation period of morphogenetic movements known as neurulation the induced neural tissue becomes organised into a hollow neural tube, the precursor of the vertebrate CNS. First, the neural ectoderm thickens to form the neural plate. Then, the lateral edges of the neural plate start to rear up in folds forming the neural folds, that gradually roll together, while the midline of the plate sinks deeper. Finally, the neural folds fuse to form the neural tube, which remains covered by a layer of ectoderm. This tube will give rise to the brain and the spinal cord, i.e., the central nervous system (CNS).

Along the line where the neural tube separates from the future epidermis, a number of ectodermal cells break loose from the epithelium to form the neural crest. These cells originate from the marginal zone between the neural plate and prospective epidermis. Neural crest cells migrate through the mesoderm to reach diverse regions within the vertebrate embryo where they subsequently give rise to a wide variety of derivatives. Neural crest cells form almost all the peripheral nervous system (PNS), including the autonomic sympathetic and parasympathetic ganglia, most of the sensory ganglia, Schwann cells and satellite cells. Moreover, they give rise to the chromaffin cells of the medular adrenal gland and the melanocytes of the skin, and in the head, many neural crest cells differentiate into connective tissues.

1.2. GANGLIA FROM THE PERIPHERAL NERVOUS SYSTEM

The peripheral nervous system is composed by ganglia from the autonomic and sensory systems and the peripheral nerves that lie outside the brain and spinal cord. The autonomic nervous system has three principal divisions: the sympathetic (or thoracolumbar), the parasympathetic (or craniosacral), and the enteric system.

1.2.1. Sensory ganglia

a) Cranial sensory ganglia: are found on five of the 12 pairs of cranial nerves. They include:

-The trigeminal ganglion (located on the V cranial nerve): it contains neurons that innervate mainly mechanoreceptors, thermoreceptors and nociceptors in the face and both oral and nasal cavities, and give sensory innervation to the teeth.

-The geniculate ganglion (present on the VII cranial nerve): it is composed of neurons that innervate the specialised receptor cells of taste buds in the anterior two-thirds of the tongue.

-The jugular ganglion (found on the proximal X cranial nerve) and the nodose ganglion (found on the distal X cranial nerve): they are in charge of conveying a variety of visceral sensory information from the pharynx, larynx, thorax and abdomen.

-The petrosal ganglion (located on the distal IX cranial nerve): it is composed of neurons that innervate taste buds in the posterior third of the tongue.

-The vestibulo-acoustic ganglion (present on the VIII cranial nerve): it contains neurons that innervate the hair cells of the organs of hearing and balance.

-The trigeminal mesencephalic nucleus: located in the midbrain, it contains proprioceptive neurons that innervate muscle spindles and tendon organs in the masticatory muscles. This is the only central nervous system site in which primary sensory neuron cell bodies that derive from the neural crest have been found. This nucleus does not belong to the PNS, although it is included in this classification because it contains primary sensory neurons.

b) Dorsal root ganglia: are located on the dorsal roots of the spinal nerves immediately adjacent to the spinal cord. Neurons in the dorsal root ganglia (DRG) convey sensory information from the skin, muscles, and joints of the limbs and trunk.

1.2.2. Sympathetic ganglia

a) The paravertebral lumbar chain: includes the superior cervical ganglion (that innervates diverse structures in the head and neck, like the salivary, lacrimal, and sweat glands, vessels, and hair follicles), the middle cervical ganglion and the stellate ganglion (they both give innervation to the heart, lungs and bronchi), and the sympathetic chain ganglia (that innervate the body wall).

b) The prevertebral ganglia: include the celiac ganglion (that innervates the esophagus and stomach), the aorticorenal ganglion (that innervates the kidneys), the superior mesenteric ganglion (that gives innervation to the small intestine), and the inferior mesenteric ganglion (that innervates the cecum, colon and pelvic organs). Finally, the adrenal medulla which contains chromaffin cells can be considered as a prevertebral ganglion as these cells are developmentally and functionally related to postganglionic sympathetic neurons.

1.2.3. Parasympathetic ganglia

These include the ciliary ganglion (that innervates vessels in the choroid layer, the pupillary constrictor and ciliary muscle), the pterygopalatine ganglion (which gives innervation to the lacrimal glands and palatal, pharyngeal and nasal mucous glands), the submandibular ganglion (which innervates the submandibular and sublingual glands, and oral mucous glands), the otic ganglion (which innervates the parotids glands and oral mucous glands), the terminal ganglia of the vagus nerve (which innervate all the viscera of the torax and abdominal cavity), and the pelvic plexus (which gives innervation to the bladder, descending colon, rectum and genitalia).

1.2.4. Enteric ganglia

The enteric nervous system (ENS) is composed of a collection of autonomic ganglia, which contain sensory neurons, interneurons, motor neurons, and astrocyte-like glia, and are arranged in interconnected plexuses distributed in the wall of the gastrointestinal tract. The two major plexuses are the submucous (or Meissner's) and the myenteric (or Auerbach's) plexuses. The ENS ganglia innervate the muscles of the gut wall and the vessels and glands of the mucosa.

1.3. ORIGINS OF THE PERIPHERAL NERVOUS SYSTEM

Sensory neurons differentiate from progenitor cells that originate from two regions of the embryonic ectoderm, the neural crest and neurogenic placodes (Noden, 1978; D'Amico-Martel and Noden, 1983; Le Douarin, 1986). The neurogenic placodes are epithelial thickenings appearing in the cephalic ectoderm of

the early vertebrate embryo. In the chicken embryo, the neurons of the ventrolateral part of the trigeminal ganglion, and the vestibulo-acoustic, geniculate, petrosal and nodose ganglia are derived from neurogenic epibranchial placodes. The neuronal cells of the dorsomedial trigeminal ganglion, the trigeminal mesencephalic nucleus, the jugular ganglion and the dorsal root ganglia are derived from different regions of the neural crest (D'Amico-Martel and Noden, 1983; Vogel, 1992). The Schwann cells and satellite cells associated with all sensory ganglia arise exclusively from the neural crest (D'Amico-Martel and Noden, 1983; Vogel, 1992).

Trunk neural crest cells give rise to the neurons and glial cells of the sympathetic and parasympathetic ganglia of the body (D'Amico-Martel and Noden, 1983; Bronner-Fraser, 1988; Le Douarin, 1980). Both neuronal and glial cells of the ENS are derived from the neural crest (NC). Enteric progenitors originate from three regions of the NC, the vagal, sacral and truncal region (Le Douarin *et al.*, 1973; 1974; Bronner-Fraser, 1988; Serbedzija *et al.*, 1991; Epstein *et al.*, 1994).

Cell lineage studies have shown that premigratory crest cells are not generally commited to single fate prior to migration (Bronner-Fraser, 1988). Their differentiation is influenced by the microenvironment of both the migratory pathway and the destination site.

2. THE NEUROTROPHIC THEORY AND APOPTOSIS

2.1. THE NEUROTROPHIC THEORY

Neurons are generated in excess during the development of the vertebrate nervous system. Approximately 50% of the neurons die shortly after their axons reach their target fields, i.e. the tissues they innervate, during a short period called "phase of naturally occurring cell death" (for review, see Davies, 1994a). This loss may be required to match the number of neurons to the size and requirements of their target fields. Neuronal death occurs by apoptosis, a descriptive name given to a genetically regulated process of cell death (Oppenheim, 1991). According to the neurotrophic hypothesis, target fields regulate the size of the neuronal populations that innervate them by producing limiting amounts of neurotrophic factors, which are essential for the survival of developing neurons. Because the supply of these factors is though to be limiting, only a proportion of the neurons are able to obtain enough trophic factor to survive. Thus, the level of trophic factor production in the target field directly influences the size of the innervating population of neurons (Davies, 1994). The neurotrophic theory was initially formulated on the basis of work on nerve growth factor (NGF), the first neurotrophic factor to be identified (Levi-Montalcini, 1987), and posteriorly generalized with the discovery of other neurotrophic factors such as BDNF (Barde *et al.*, 1982).

In support of the neurotrophic hypothesis, certain populations of developing neurons are dependent on NGF for survival *in vitro* and *in vivo*. Administration of anti-NGF antibodies during their phase of target field innervation increases neuronal death, whereas exogenous NGF enhances the number of surviving neurons that would otherwise die (Levi-Montalcini, 1987). Recent data obtained from mutant mice with targeted disruption of either the NGF gene or the NGF receptor tyrosine kinase gene have confirmed the dependency of these neurons on NGF for survival during development (Crowley *et al.*, 1994; Smeyne *et al.*, 1994). Furthermore, NGF is synthesized in the tissues innervated by these neurons coinciding with the beginning of target field innervation (Davies *et al.*, 1987; Korsching and Thoenen, 1988). The level of NGF expression in these tissues is found to be proportional to their final innervation density (Harper and Davies, 1990).

It is currently believed that multiple neurotrophic factors may cooperate in regulating the survival of neurons during development and in some cases these factors are supplied using mechanisms apparently in contradiction with the neurotrophic theory. Thus, it has been shown that neurons can obtain neurotrophic factors via anterograde, autocrine, and paracrine routes (for reviews, see Oppenheim, 1991; Davies, 1996a), although the physiological relevance of these findings in the regulation of neuronal survival *in vivo* remains unclear.

2.2. APOPTOSIS

Apoptosis or programmed cell death is a genetically controlled mechanism used by cells to commit suicide in response to a variety of stimuli. It plays a major role in several tissues, including the nervous system, during development to ensure a proper matching between different interacting cell populations. The execution of this death program is often associated with characteristic morphological and biochemical changes that distinguishes it from necrosis; the nucleus and cytoplasm condense, and the dying cell fragments into membrane-bound apoptotic bodies that are rapidly phagocytosed by surrounding macrophages (Wyllie *et al.*, 1980). The process of apoptosis is regulated through the expression of an increasingly discovered number of genes, conserved during evolution from nematodes and viruses to mammals. Some gene products are activators of apoptosis, whereas others are inhibitors (for review, see Davies, 1995).

A least fifteen genes encoding proteins that either inhibit or accelerate cell death compose the Bcl-2 family. The founder of the family, Bcl-2, is a proto-oncogene that was originally identified at the breakpoint of translocations commonly occurring in human B cell follicular lymphoma (Bakhshi *et al.*, 1985; Tsujimoto *et al.*, 1985; Cleary *et al.*, 1986) and encodes a membrane-associated protein that enhances cell survival by preventing cell death (Vaux *et al.*, 1988). The subgroup of vertebrate gene products that inhibit apoptosis is composed of: Bcl-2, Bcl-x_L, Bcl-w, Mcl-1 and A1, whereas the accelerators of cell death include: Bcl-x_S, Bax, Bad, Bak and Nbk/Bik1 (for review, see Rao and White, 1997). A striking feature of the Bcl-2 family members is their ability to homodimerize and heterodimerize

through the conserved regions, BH1, BH2 and BH3 (Bcl-2 homology regions 1, 2 and 3). The level of expression of pro-apoptotic and anti-apoptotic proteins and the interactions between them seem to regulate cell death (Rao and White, 1997). Furthermore, post-translational phosphorylation of some Bcl-2 family members is also involved in controlling apoptosis (for review, see Gajewski and Thompson, 1996).

Another family of proteins that regulates cell death is the interleukin-1ß-converting enzyme (ICE) family of cysteine proteases, also termed caspases. They are involved in a cascade of proteolytic events and, upon activation during apoptosis, induce cellular disassembly as a result of cleavage of numerous cellular substrates (Fraser and Evan, 1996, Rao and White, 1997; Martins and Earnshaw, 1997).

Whereas the Bcl-2 family of proteins is in control of apoptosis, the ICE family of proteins is involved in the execution of apoptosis. It has been recently demonstrated that the Bcl-2 family of proteins regulate caspase activation by translocation of cytochrome C and a protease, AIF (apoptosis-inducing factor) from the mitochondria to the cytosol, functionally linking the two families (for reviews, see Golstein, 1997; Martins and Earnshaw, 1997).

Some signalling molecules, like neurotrophic factors, induce a survival response, whereas others, like the Fas ligand and TNF, promote apoptosis. A functional connection between the intracellular signalling pathway mediated by Fas or TNFR-I and the activation of the cell death machinary has been recently reported (Fraser and Evan, 1996). However, the intracellular events mediated by the neurotrophic factor receptors that lead to cell survival are mostly unknown.

The survival of neurons during the development of the vertebrate nervous system is mainly promoted by members of three groups of neurotrophic factors: the family of neurotrophins, the family of neuropoietic cytokines and the family of TGF-ß-related proteins.

3. THE FAMILY OF NEUROTROPHINS AND THEIR RECEPTORS

The neurotrophins are the best characterized family of neurotrophic factors. The family comprises six highly homologous proteins; nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), neurotrophin-4/5 (NT-4/5) and neurotrophin-6 (NT-6). These proteins regulate numerous events during the development of the vertebrate nervous system including neuroblast proliferation, neuronal survival and differentiation, axonal branching, and synaptic function (Davies, 1994b; Snider, 1994; Thoenen, 1995). It has been suggested that the NGF gene appeared more recently in evolution than BDNF and NT-3, probably as a result of gene duplication and diversification by mutations (Barde, 1994). Neurotrophins transduce their signal into the cell through binding to a receptor complex composed of two transmembrane proteins, a specific high-affinity receptor tyrosine kinase (Trk) and a common low affinity receptor ($p75^{NTR}$) (for review, see Snider, 1994).

3.1. MEMBERS OF THE NEUROTROPHIN FAMILY

3.1.1. Nerve growth factor

Nerve growth factor (NGF) is a protein that was initially purified and characterized from the submandibular gland of the adult male mouse, where it exists in inexplicably large amounts, on the basis of its ability to induce fibre outgrowth from sensory and sympathetic ganglia both *in vivo* and *in vitro* (Cohen, 1960). The determination of the mouse NGF amino acid sequence (Angeletti and Bradshaw, 1971) led to the cloning of the mouse NGF cDNA (Scott *et al.*, 1983). Subsequently, human, bovine and chicken NGF genes have been cloned (Ullrich *et al.*, 1983; Meier *et al.*, 1986; Ebendal *et al.*, 1986). The mouse NGF gene encodes a 307 amino acid precursor polypeptide that is processed to release a 118-residue glycosylated mature protein (Berger and Shooter, 1977; Scott *et al.*, 1983; Edwards *et al.*, 1988). Analysis of the three-dimensional structure of mature NGF has revealed that it contains a motif of three intrachain disulphide bridges, known as the "cysteine knot", and two antiparallel ß strands. Non-covalent homodimerization

through extensive hydrophobic interactions gives rise to biologically active NGF (McDonald *et al.*, 1991).

Several *in vitro* and *in vivo* studies have provided evidence that NGF is a survival factor for developing sympathetic neurons, certain kinds of sensory neurons and basal forebrain cholinergic neurons (Chun and Patterson, 1977; Hamburger *et al.*, 1981; Davies and Lindsay, 1985; Levi-Montalcini, 1987; Hartikka and Hefti, 1988; for reviews, see Barde, 1989; Davies, 1994b). Furthermore, NGF mRNA is expressed in the target tissues of these populations of neurons. For example, NGF synthesis in developing mouse skin coincides with the onset of target field innervation by NGF-dependent trigeminal ganglion neurons (Davies *et al.*, 1987). NGF is also present in embryonic mouse heart and submandibular gland coinciding with their innervation by sympathetic neurons (Korsching and Thoenen, 1988). NGF mRNA is found in several regions of the brain including, rat hippocampus, neocortex and olfactory bulb, where it is expressed at high levels (Korsching *et al.*, 1985; Whittemore *et al.*, 1986; Maisonpierre *et al.*, 1990a), and chicken optic tectum and cerebellum (Ebendal *et al.*, 1986).

Mice with targeted disruption of the NGF gene have been generated to facilitate the study of the actions of NGF in the developing nervous system *in vivo*. Homozygous mice fail to respond to noxious mechanical stimuli due to an impaired nociceptive system (Crowley *et al.*, 1994). These mice exhibit a dramatic depletion in cell numbers in dorsal root, trigeminal and sympathetic ganglia. The neuronal loss in DRG is selective and involves small-diameter neurons that are thought to mediate pain and thermal receptive functions. In contrast, there is no marked deficit in any neuronal population within the CNS of NGF knockout mice indicating that NGF is not an essential survival factor in the CNS (Crowley *et al.*, 1994).

3.1.2. Brain-derived neurotrophic factor

Brain-derived neurotrophic factor (BDNF) is a 12.3 kDa basic protein that was first purified from adult pig brain and identified as a survival factor for E10 chicken DRG neurons in culture (Barde *et al.*, 1982). Data obtained from partial amino acid sequencing was used for cloning the pig BDNF cDNA (Leibrock *et al.*, 1989). BDNF, like NGF, is synthesized as a precursor protein. Following

cleavage and glycosylation the 252 amino acid BDNF precursor gives rise to a 119-residue mature polypeptide. Furthermore, BDNF has approximately 50% identity with NGF and contains the six cysteine residues involved in forming the "cysteine knot" motif found in its homologue. Biologically active BDNF exists as a tighly associated homodimer (Radziejewski *et al.*, 1992). BDNF has subsequently been cloned from other species including human (Jones and Reichardt, 1990; Maisonpierre *et al.*, 1991), mouse (Hofer *et al.*, 1990) and rat (Maisonpierre *et al.*, 1991). The gene encoding human BDNF is localized to chromosome 11, band p13 (Maisonpierre *et al.*, 1991).

It has been demonstrated that BDNF enhances the survival and differentiation of several classes of CNS neurons in culture, including embryonic rat basal forebrain cholinergic neurons, mesencephalic dopaminergic neurons, motoneurons, hippocampal neurons, cerebellar granule cells, and retinal ganglion cells (Alderson *et al.*, 1990; Hyman *et al.*, 1991; Knüsel *et al.*, 1991; Oppenheim *et al.*, 1992; for review, see Korsching, 1993). In the peripheral nervous system, BDNF promotes the survival of embryonic neural crest-derived (dorsomedial part of the trigeminal ganglion, trigeminal mesencephalic nucleus, jugular and dorsal root ganglion) and placode-derived (ventrolateral part of the trigeminal ganglion, geniculate, petrosal, vestibular and nodose ganglion) neurons (Davies *et al.*, 1986a; Barde *et al.*, 1987; Hofer and Barde, 1988).

BDNF mRNA expression is largely restricted to the CNS. In general, low level restricted expression of BDNF mRNA in the embryonic brain gives way to more widespread, higher level of expression as the CNS matures. In the adult CNS, BDNF mRNA is found in substantial amounts in the cortex, hippocampus, and cerebellum, and in lower levels in the midbrain, hindbrain, striatum, olfactory bulb and spinal cord (Leibrock *et al.*, 1989; Hofer *et al.*, 1990). In the PNS, BDNF mRNA is found in neurons of the dorsal root and trigeminal ganglia, where it is expressed by NGF-dependent neurons, and sympathetic ganglia, suggesting a possible autocrine and/or paracrine mechanism (Robinson *et al.*, 1996; Schecterson and Bothwell, 1992). Outside of the nervous system, BDNF mRNA has been shown to be expressed at low levels in the muscle, heart, skin and lung (Maisonpierre *et al.*, 1990a; Schecterson and Bothwell, 1992).

Homozygous mice with a null mutation of the BDNF gene develop symptoms of nervous system dysfunction, including ataxia. These mice display a substantial reduction in the number of neurons of the trigeminal, geniculate, vestibular and petrosal-nodose ganglia, whereas the CNS develops with no apparent gross structural abnormalities (Jones *et al.*, 1994; Ernfors *et al.*, 1994a). This indicates that BDNF is an essential factor for the survival of several populations of cranial and spinal sensory neurons, whereas it does not appear to be essential for the development of sympathetic, dopaminergic and motoneurons (Jones *et al.*, 1994; Ernfors *et al.*, 1994a).

3.1.3. Neurotrophin-3

Neurotrophin-3 (NT-3) is the third member of the neurotrophin family. It was identified by using degenerate oligonucleotides derived from conserved regions between NGF and BDNF to screen a mouse cDNA library. The mature mouse NT-3 is a 13.6 kDa basic protein and consists of 119 amino acids that originate from a 258-residue precursor polypeptide. Mature NT-3 is structurally related to both BDNF and NGF and shares about 50-60% amino acid identity with these two neurotrophic factors, including all six cysteine residues which in NGF and BDNF are involved in the formation of disulphide bridges (Hohn *et al.*, 1990). Biologically active NT-3, like NGF and BDNF, is a homodimer (Radziejewski *et al.*, 1992). The cloning of human and rat NT-3 has subsequently been reported (Rosenthal *et al.*, 1990; Ernfors *et al.*, 1990; Maisonpierre *et al.*, 1990b; 1991) and the gene encoding human NT-3 has been localized to chromosome 12, band p13 (Maisonpierre *et al.*, 1991).

NT-3 is mainly involved in events during early development. For example, it stimulates sympathetic neuroblast proliferation by promoting precursor survival, and induces the differentiation of neural tube precursors into motoneurons (DiCicco-Bloom *et al.*, 1993; Averbuch *et al.*, 1994). Furthermore, NT-3 transiently supports the survival of neural crest-derived sensory and sympathetic neurons in early stages of development before they switch their dependence to NGF (Buchman and Davies, 1993; Buj-Bello *et al.*, 1994; Birren *et al.*, 1993). Moreover, NT-3 sustains the survival of subpopulations of embryonic sympathetic, nodose, trigeminal and dorsal root ganglion, and mesencephalic trigeminal nucleus neurons

in culture (Ernfors *et al.*, 1990; Maisonpierre *et al.*, 1990b; Hohn *et al.*, 1990; Rosenthal *et al.*, 1990). In the CNS, NT-3 promotes the survival of embryonic motoneurons and noradrenergic neurons *in vitro* (Henderson *et al.*, 1993; Friedman *et al.*, 1993) and prevents 6-hydroxydopamine-induced degeneration of adult noradrenergic neurons of the locus coeruleus *in vivo* (Arenas and Persson, 1994). NT-3 also increases oligodendrocyte proliferation in the optic nerve during development (Barres *et al.*, 1994).

In accordance with its role in early development, NT-3 is highly expressed in immature regions of the CNS, where the processes of proliferation, migration, and differentiation of neuronal precursors are occurring, and its expression decreases dramatically in these regions with maturation (Maisonpierre *et al.*, 1991). NT-3 mRNA is widely expressed in the adult both in the nervous system and peripheral tissues. In the brain, levels of NT-3 mRNA are greatest in the cerebellum and hippocampus. In the periphery, areas of notable expression include heart, skin, gut, muscle, liver, kidney, lung and spleen (Hohn *et al.*, 1990; Rosenthal *et al.*, 1990).

The physiological relevance of NT-3 during development has been revealed by analysis of mutant mice with targeted disruption of the NT-3 gene (Ernfors *et al.*, 1994b). Homozygous mice display a substantial neuronal cell loss in all sensory ganglia, trigeminal mesencephalic nucleus and sympathetic superior cervical ganglion. Importantly, the main components of the propioceptive system, namely the muscle spindles and Golgi tendon organs, are missing together with large-diameter propioceptive DRG neurons (Ernfors *et al.*, 1994b).

3.1.4. Neurotrophin-4/5

Taking advantage of the structural similarity between NGF, BDNF and NT-3, a fourth member of this protein family was identified and termed neurotrophin-4/5 (NT-4/5). Xenopus and viper NT-4 encode a 236 amino acid precursor polypeptide that is processed to a 123-residue mature protein with 50%-60% amino acid identity to its homologues, NGF, BDNF, and NT-3, including conservation of all six cysteine residues common to the entire family (Hallböök *et al.*, 1991). Homologous human and rat NT-4 clones, initially designated NT-5, were subsequently isolated and share about 65% amino acid identity with their Xenopus

counterpart (Berkemeier *et al.*, 1991; Ip *et al.*, 1992a). It is currently believed that Xenopus NT-4 and mammalian NT-5 are the same molecule and are referred as NT-4/5.

In the PNS, human recombinant NT-4/5 supports transiently the survival *in vitro* of embryonic rodent trigeminal and jugular ganglion neurons at early stages of target field innervation, and mouse nodose ganglion neurons during the phase of naturally occurring cell death. Significantly, all of these neurons are also BDNF responsive during the same developmental periods. In contrast, the survival-promoting effect of NT-4/5 on embryonic chicken neurons is very limited (Davies *et al.*, 1993a; Ibáñez *et al.*, 1993). This difference in specificity suggests that NT-4/5 may be a mammalian neurotrophic factor; alternatively, the structure of NT-4/5 may not be well conserved between mammals and birds. In the CNS, it has been shown that NT-4/5 sustains the *in vitro* survival of embryonic rat motoneurons (Henderson *et al.*, 1993), and cholinergic basal forebrain and noradrenergic locus coereulus neurons (Friedman *et al.*, 1993).

NT-4/5 may be a target-derived neurotrophic factor for trigeminal ganglion neurons, since NT-4/5 mRNA has been found to be expressed in the developing rat whisker pad, their major peripheral target tissue, during the phase of naturally occurring cell death of these neurons (Ibáñez *et al.*, 1993). NT-4/5 mRNA is also present in several other embryonic and adult rat tissues, including brain, heart, muscle, lung, kidney, thymus, ovary and testis (Berkemeier *et al.*, 1991; Timmusk *et al.*, 1993).

It has been demonstrated that mice with a targeted disruption of the NT-4 gene exhibit a loss of sensory neurons in the nodose-petrosal and geniculate ganglia, whereas sympathetic neurons of the superior cervical ganglion, facial motoneurons and midbrain dopaminergic neurons are unaffected. Furthermore, the phenotype of mice lacking both BDNF and NT-4 is similar that of BDNF or NT-4 knockouts alone, although there is a more severe neuronal reduction in sensory ganglia (Conover *et al.*, 1995; Liu *et al.*, 1995).

3.1.5. Neurotrophin-6

Neurotrophin-6 (NT-6) represents the most recently isolated member of the neurotrophin family, and was cloned from the teleost fish Xiphophorus maculatus (Götz *et al.*, 1994). In accordance with the features of all known neurotrophins, NT-6 is synthesized as a precursor polypeptide of 286 amino acids that upon proteolytic cleavage gives rise to a 143-residue mature basic protein with a molecular mass of 15.9 kDa that contains the six cysteines conserved in all neurotrophins known so far. In contrast to the other members, NT-6 has not been found as a soluble protein in the medium of producing cells and it is likely to be bound to proteoglycans of the cell surface and/or extracellular matrix (Götz *et al.*, 1994).

It has been demonstrated that NT-6, like NGF, promotes the survival of sympathetic and a subpopulation of dorsal root ganglion neurons, whereas it has no effect on ciliary and nodose neurons (Götz *et al.*, 1994). Analysis of NT-6 mRNA expression in the teleost fish has revealed its presence in embryonic and adult cerebellum, and several other adult tissues, including gill, liver, eye, skin, spleen, heart and skeletal muscle (Götz *et al.*, 1994).

3.2. RECEPTORS OF THE NEUROTROPHIN FAMILY

The various members of the neurotrophin family bind to two kinds of unrelated cell surface receptors, named $p75^{NTR}$ and Trk (pronounced track). $p75^{NTR}$ and Trk were first identified by cross-linking studies using radioiodinated NGF (Sutter *et al.*, 1979) and found to interact with NGF with low and high affinity , respectively (for review, see Meakin and Shooter, 1992).

The low affinity receptor for neurotrophins, termed $p75^{NTR}$, has been cloned in different species including human, rat and chicken (Chao *et al.*, 1986; Radeke *et al.*, 1987; Large *et al.*, 1989). $p75^{NTR}$ is a single 75 kDa transmembrane glycoprotein that contains a long extracellular domain with four cysteine repeats that are responsible for ligand recognition (Welcher *et al.*, 1991; Yan *et al.*, 1991). These cysteine repeats are common to members of a family of cell surface receptors that includes the Fas antigen and type I and II receptors for tumor necrosis factor (TNFR-I and TNFR-II) (Nagata and Golstein, 1995). The shorter intracellular domain of $p75^{NTR}$ contains a small segment homologous to the death domain of Fas and TNFRI (Nagata and Golstein, 1995). Although it was first described as a low affinity receptor for NGF, it has been demonstrated that $p75^{NTR}$ binds with similar low affinity to most neurotrophins, including BDNF, NT-3 and NT-4/5 (for review, see Davies, 1994c).

Ligand specificity of neurotrophin receptors is determined by the high affinity receptor component, the receptor tyrosine kinase (Trk). Trk is a proto-oncogene that was first identified as a human oncogene resulting from DNA rearrangement between truncated tropomyosin and protein tyrosine kinase sequences (Martin-Zanca *et al.*, 1986; 1989). It encodes a 140 kDa transmembrane glycoprotein that contains a cytoplasmic tyrosine kinase domain, and is referred to as $gp140^{trkA}$ or TrkA (Martin-Zanca *et al.*, 1989). TrkA is the founder of a family of homologous receptor tyrosine kinases that also includes TrkB and TrkC. TrkB, also named $gp145^{trkB}$, was cloned from a mouse brain cDNA library using a human TrkA probe under low stringency conditions. TrkB encodes a 145 kDa transmembrane protein tyrosine kinase that shares about 70% homology with TrkA (Klein *et al.*, 1989). TrkC, also designated $gp145^{trkC}$, was originally cloned by screening a porcine brain

cDNA library with a human TrkA probe. TrkC encodes a 145 kDa transmembrane glycoprotein with tyrosine kinase activity that shares about 65% homology with TrkA and TrkB (Lamballe *et al.*, 1991). NGF, BDNF and NT-3 bind preferentially to TrkA, TrkB and TrkC, respectively (Kaplan *et al.*, 1991a; 1991b; Klein *et al.*, 1991a; 1991b; Soppet *et al.*, 1991; Squinto *et al.*, 1991; Lamballe *et al.*, 1991). NT-4/5 signals through TrkB (Berkemeier *et al.*, 1991; Klein *et al.*, 1992). Furthermore, it appears that some neurotrophins can interact with more than one neurotrophin receptor. Thus, NT-3 can also bind to TrkA and TrkB (Klein *et al.*, 1991b; Soppet *et al.*, 1991; Squinto *et al.*, 1991), and NT-4/5 can activate TrkA (Berkemeier *et al.*, 1991). The receptor specificities of NT-6 have not been defined so far.

Interestingly, several isoforms of the receptor tyrosine kinases have been found, although their physiological relevance is not fully understood. The TrkA gene encodes two protein isoforms that differ in the presence of six amino acid residues located in their extracellular domain (Barker *et al.*, 1993). The TrkB gene generates several different transcripts that, apart from the full length gp145^{trkB} receptor, encode two truncated receptors that lack the entire intracellular catalytic kinase domain (Klein *et al.*, 1990; Middlemas *et al.*, 1991) and several other isoforms that contain deletions in the leucine-rich motifs of the extracellular domain (Ninkina *et al.*, 1997). The TrkC gene encodes at least four distinct protein isoforms; the full length gp145^{trkC} receptor, two isoforms with different insertions in the tyrosine kinase domain and a truncated receptor that lack the intracellular tyrosine kinase domain (Valenzuela *et al.*, 1993; Tsoulfas *et al.*, 1993).

In order to analyse the physiological relevance of the neurotrophin receptors during development, mutant mice lacking either the TrkA, TrkB or TrkC genes have been generated by homologous recombination (Klein *et al.*, 1993; 1994; Smeyne *et al.*, 1994). In general, the phenotype of mice carrying a null mutation in each neurotrophin receptor gene is similar to that observed for its ligand (Crowley *et al.*, 1994; Jones *et al.*, 1994; Ernfors *et al.*, 1994a; 1994b). Mice lacking TrkA are insensitive to pain and display a substantial loss in the number of neurons of the trigeminal, dorsal root and sympathetic ganglia, whereas cholinergic basal forebrain neurons are not affected by the absence of functional TrkA/gp140^{trkA} (Smeyne *et al.*, 1994). In mice homozygous for targeted disruption of the TrkB/gp145^{trkB} gene,

there is a marked reduction in trigeminal and dorsal root ganglion neurons (Klein *et al.*, 1993). Finally, disruption of the TrkC/gp145^{trkC} gene eliminates Ia muscle afferent projections to spinal motor neurons and reduces the number of large myelinated axons in the dorsal root and posterior columns of the spinal cord, in addition to eliminating a subpopulation of dorsal root ganglion neurons (Klein *et al.*, 1994).

Although it is clear that each neurotrophin can interact independently with different affinity with p75^{NTR} and specific Trk receptors, the role that p75^{NTR} exerts in the receptor complex for neurotrophin signalling remains to be clarified. It has been shown that p75^{NTR}-deficient mice do not display significant neuronal loss in the peripheral nervous system, but have a marked decrease in the sensory innervation of the skin (Lee *et al.*, 1992). Whereas p75^{NTR} does not seem essential for neurotrophin signalling, it has been proposed that it may increase the affinity of receptors for neurotrophin binding during competition for limited concentrations of trophic factors and be involved in the discrimination between different, but structurally related, members of the neurotrophin family (for review, see Chao and Hempstead, 1995). Two models for p75^{NTR}-Trk interaction have been proposed. In a conformational model, p75^{NTR} would interact in the absence of ligand with Trk to change its conformation and create a high affinity binding. In the presentation model, the neurotrophin would bind first to p75^{NTR} and would either increase the local concentration of the ligand or transfer the neurotrophin to the Trk receptor component (Chao and Hempstead, 1995). To further complicate the understanding of p75^{NTR} function, it has recently been shown that it can induce apoptosis in certain kind of cells such as retinal cells and oligodendrocytes. It is possible that p75^{NTR} may achieve this by activating sphingomyelinase and increasing ceramide production, and/or activating gene transcription via nuclear factor κ B (Frade *et al.*, 1996; Cassacia-Bonnefil *et al.*, 1996; for reviews, see Davies, 1996b; Carter and Lewin, 1997).

4. THE FAMILY OF NEUROPOIETIC CYTOKINES AND THEIR RECEPTORS

Neuropoietic cytokines are a group of distantly related proteins with extensive pleiotropy in the nervous, hematopoietic and immune systems and other non-neuronal tissues. Although they share less than 30% sequence homology at the amino acid level, their secondary structure adopts a similar four antiparallel helix bundle that resembles that determined for growth hormone (Bazan, 1991). Furthermore, they share a similar gene structure, suggesting that these cytokines evolved from a common ancestral gene (Bruce *et al.*, 1992). Members of this family bind to related multisubunit receptors that contain a common transducing component (Sato and Miyajima, 1994). This family of proteins is composed of ciliary neurotrophic factor (CNTF), the most extensively studied neuropoietic cytokine in the nervous system, growth-promoting activity (GPA), leukaemia inhibitory factor (LIF), interleukin-6 (IL-6), oncostatin M (OSM), cardiotrophin-1 (CT-1), interleukin-11 (IL-11) and granulocyte-colony stimulating factor (G-CSF). Outlined below is an account of the members of this family of proteins that have known functions or activities in the nervous system.

4.1. MEMBERS OF THE NEUROPOIETIC FAMILY OF CYTOKINES

4.1.1. Ciliary neurotrophic factor

Ciliary neurotrophic factor (CNTF) was initially identified as a neurotrophic activity present in eye tissues that was able to promote the *in vitro* survival of parasympathetic neurons of the chicken ciliary ganglion (Adler *et al.*, 1979). CNTF was purified from extracts of embryonic chicken intraocular tissues (Barbin *et al.*, 1984) and subsequently from adult rat sciatic nerve, which is a rich and easily accessible source for this factor (Manthorpe *et al.*, 1986), and shown to be an acidic protein with an estimated molecular mass of 20-24 kD. Highly purified CNTF isolated from sciatic nerve was used to determine its partial amino acid sequence, which led to the cloning of rabbit and rat CNTF cDNAs (Lin *et al.*, 1989; Stöckli *et al.*, 1989). These cDNAs encode proteins of 199 and 200 amino acids,

respectively. In contrast to other neurotrophic factors, CNTF appears to be a cytosolic protein, since it lacks a signal peptide sequence for secretion and consensus sequences for glycosylation. In accordance with this, CNTF is not released into the medium from transfected HeLa or COS cells (Lin *et al.*, 1989; Stöckli *et al.*, 1989). It is unclear whether CNTF could be released from the cytoplasm either after a microtrauma that disrupts the integrity of the plasma membrane of CNTF-synthesizing cells, or by an unknown secretion mechanism.

CNTF is a trophic factor with widespread biological activity that affects mainly neuronal cells, but also non-neuronal cells (for review, see Sendtner *et al.*, 1994). It supports the survival of several populations of PNS and CNS neurons in culture. For example, neurons isolated from chicken ciliary, nodose, trigeminal, dorsal root and sympathetic lumbar ganglia respond *in vitro* to CNTF (Barbin *et al.*, 1984; Manthorpe and Varon, 1985). Similarly, CNTF provides trophic support to spinal motoneurons (Arakawa *et al.*, 1990), hippocampal neurons (Ip *et al.*, 1991) and Purkinje cells in culture (Lärkfors *et al.*, 1994). Despite the survival-promoting effect of CNTF on multiple neuronal types, *in ovo* administration of CNTF only protects chicken motoneurons from naturally occurring cell death, whereas sensory, sympathetic and nodose neurons are unaffected by this treatment (Oppenheim *et al.*, 1991). CNTF also prevents the axotomy-induced neuronal degeneration of a variety of neurons including facial (Sendtner *et al.*, 1990) and spinal motoneurons (Li *et al.*, 1994; Vejsada *et al.*, 1995), neurons in the anterior thalamic nuclei (Clatterbuck *et al.*, 1993) and dopaminergic neurons of the substantia nigra (Hagg and Varon, 1993). Interestingly, it has been demonstrated that peripheral nerve axotomy enhances the retrograde axonal transport of CNTF by dorsal root ganglion neurons and spinal motoneurons (Curtis *et al.*, 1993).

Apart from its survival-promoting effects on neuronal cells, CNTF also displays other actions on non-neuronal cells of the nervous system. For example, oligodendrocyte-type-2-astrocyte (O-A2) progenitor cells differentiate *in vitro* into type-2 astrocytes in the presence of CNTF (Hughes *et al.*, 1988) and microglial cells differentiate into cells with a reactive phagocytotic phenotype (Sendtner *et al.*, 1994). Furthermore, CNTF inhibits the proliferation and induces cholinergic differentiation of developing sympathetic neurons (Ernsberger *et al.*, 1989a; Saadat *et al.*, 1989). CNTF acts only on limited targets outside the nervous system. It has

been shown that it exerts myotrophic effects on denervated skeletal muscle *in vivo*, attenuating the atrophy and functional changes associated with denervation (Helgren *et al.*, 1994).

Taken together these data suggest that CNTF could play an important role in the nervous system, both during development and under pathological conditions, with limited additional roles in other tissues. Surprisingly, analysis of homozygous mutant mice with targeted disruption of the CNTF gene has revealed that a lack of CNTF has no apparent effects on the nervous system during embryonic development. However, CNTF appears to be essential for the maintenance of adult motoneurons, since they degenerate progressively during the postnatal period in its absence (Masu *et al.*, 1993). This finding is in agreement with the observation that CNTF mRNA expression is absent in whole rat embryos at E9 and in both brain and hindlimbs at E18 (Stöckli *et al.*, 1991). In contrast, high levels of CNTF mRNA are widely expressed in the adult rat nervous system, in particular in the sciatic and optic nerves, olfactory bulb and spinal cord. Lower levels of expression are detected in the brain stem, cerebellum, septum, hippocampus, striatum, and midbrain (Stöckli *et al.*, 1991; Dobrea *et al.*, 1992; Ip *et al.*, 1993a). In these tissues, CNTF is mainly produced by glial cells. CNTF mRNA expression could not be detected in tissues outside of nervous system in adult rats (Stöckli *et al.*, 1989; Dobrea *et al.*, 1992).

It has been suggested that CNTF might act as a lesion factor under pathological conditions. In agreement with this hypothesis, most newborn rodent motoneurons undergo cell death following axotomy coinciding with the period when CNTF is not expressed in the peripheral nerve. At the end of the first post-natal week Schwann cells surrounding peripheral axons start to synthesize CNTF and motoneurons become more resistant to nerve lesions (Stöckli *et al.*, 1989; Sendtner *et al.*, 1990; Li *et al.*, 1994). CNTF very effectively prevents axotomy-induced cell death of newborn rat facial motoneurons (Sendtner *et al.*, 1990) and degeneration of motoneurons in mutant mice homozygous for progressive motor neuropathy (pmn) (Sendtner *et al.*, 1992b). It has been shown that CNTF synthesis is regulated by a neuron-Schwann cell interactive signal, since CNTF remains expressed in Schwann cells in the proximal segment of the sciatic nerve after transection, whereas its expression drops markely in the distal portion of the

sectioned nerve, where the remaining CNTF is released at extracellular sites, until regeneration takes place (Sendtner *et al.*, 1992a). The potential role of CNTF as a lesion factor may also apply to the adult rat CNS, where, in contrast to the peripheral nervous system, the synthesis of CNTF is up-regulated by reactive astrocytes in the wound after injury (Ip *et al.*, 1993a). Despite all of these findings, the role of CNTF *in vivo* remains unclear and will need more efforts to be clarified.

4.1.2. Growth-promoting activity

Growth-promoting activity (GPA) activity was originally characterized in chicken eye extracts (Nishi and Berg, 1981). The purification of GPA from adult chicken sciatic nerves led to the determination of its partial amino acid sequence, and revealed it to be a protein homologous to CNTF (Eckenstein *et al.*, 1990). Subsequently, GPA cDNA was cloned from an embryonic eye cDNA library (Leung *et al.*, 1992). GPA shares 50% amino acid identity with mammalian CNTFs, suggesting that this protein could be the chicken homologue of CNTF. However, controversial data obtained from a limited number of experiments comparing GPA and CNTF function and expression could indicate that they are different molecules. GPA, like CNTF, lacks an hydrophobic amino-terminal sequence for secretion. However, in contrast to CNTF, GPA is released in a biologically active form into the medium surrounding transfected cells by an unkown mechanism (Leung *et al.*, 1992). *In vitro*, GPA and CNTF both inhibit the proliferation of chicken sympathetic neuroblasts and induce a cholinergic phenotype, with GPA being more potent than CNTF (Heller *et al.*, 1993). GPA, like CNTF, can promote the survival of chicken E8 ciliary, E10 DRG and E11-12 sympathetic neurons in culture. However, in contrast to CNTF, GPA also supports E8 DRG neuron survival *in vitro* (Eckenstein *et al.*, 1990). Moreover, GPA and CNTF seem to interact with similar but not identical receptors, since GPA competes less efficiently than CNTF for the binding of ^{125}I-labelled CNTF on chicken sympathetic neurons (Heller *et al.*, 1993). Finally, GPA mRNA, unlike CNTF, is expressed in the chicken embryo during development. In particular, GPA is found in the embryonic uveal tract, the target tissue for ciliary ganglion neurons, and in the embryonic sciatic nerve (Leung *et al.*, 1992).

Taken together, these data suggest that GPA and CNTF might be distinct neurotrophic factors. Thus, it remains to be clarified whether a mammalian homologue to GPA exists. Alternatively, it is possible that the same protein exhibits different actions, in different species and at different times.

4.1.3. Leukaemia inhibitory factor

Cholinergic differentiation factor (CDF), is a pleiotropic cytokine originally purified from rat heart cell conditioned medium on the basis of its ability to induce cholinergic differentiation of rat sympathetic neurons in culture (Fukada, 1985). Subsequently, leukaemia inhibitory factor (LIF) was purified on the basis of its ability to regulate the growth and differentiation of the M1 myeloid cell line (Gearing *et al.*, 1987). It has been demonstrated that CDF and LIF correspond to the same protein (Yamamori *et al.*, 1989). LIF exhibits multiple actions in the nervous and hematopoietic systems (for reviews, see Hall and Rao, 1992; Patterson, 1994). The effects of LIF in the nervous system are in general very similar to that of CNTF. LIF regulates the transmitter phenotype and expression of several neuropeptides in cultured sympathetic and sensory neurons (Yamamori *et al.*, 1989; Nawa *et al.*, 1991; Bazan, 1991; Fan and Katz, 1993). Furthermore, LIF stimulates the differentiation of neural crest cells into sensory-like neurons and enhances the survival of several populations of embryonic and postnatal sensory neurons in culture (for review, see Horton *et al.*, 1996). In addition to its effects on sensory neurons, LIF also exerts trophic effects on cultured spinal motoneurons (Martinou *et al.*, 1992) and promotes the differentiation of neuronal and astrocytic precursors cells in the developing spinal cord (Richards *et al.*, 1996). However, in contrast to the *in vitro* actions of LIF on several kinds of neurons, mice with targeted disruption of the LIF gene do not exhibit any developmental abnormality in these neuronal populations, but display alterations in response to injury (Stewart *et al.*, 1992). It has been shown that LIF is transported retrogradely by DRG neurons and spinal motoneurons under normal conditions and its transport is increased after sciatic nerve injury. Interestingly, LIF mRNA expression is present in the peripheral nerve and up-regulated at the site of lesion and in the distal portion of the nerve after axotomy (Curtis *et al.*, 1994; Banner *et al.*, 1994). These findings

suggest that LIF, like CNTF, may play a role *in vivo* in neuronal maintenance and axonal regeneration following injury.

4.1.4. Interleukin-6

Interleukin-6 (IL-6) is a multifunctional cytokine produced by a variety of cells that was originally characterized as a factor with an important role in inflamation and in response to infection (Hirano, 1991). In addition to its functions in the immune system, IL-6 is involved in physiological and pathological events that occur in the developing and adult nervous system. It supports the survival of forebrain cholinergic, midbrain catecholaminergic, spinal cord acethylcholinesterase-positive, and nodose and trigeminal ganglion neurons (Hama *et al.*, 1989; Kushima *et al.*, 1992; Kushima and Hatanaka, 1992; Horton *et al.*, 1996). Furthermore, it induces cholinergic differentiation and expression of neuropeptides such as substance P in cultured sympathetic neurons (Fann and Patterson, 1994). Although IL-6 mRNA is not present in intact nerves, its expression is rapidly up-regulated in degenerating nerves after axotomy (Reichert *et al.*, 1996). IL-6 transcripts are also detected in DRG neurons after nerve transection (Murphy *et al.*, 1995). These findings suggest that IL-6, like LIF and CNTF, is a cytokine with an important role during neuronal regeneration following axotomy.

4.1.5. Oncostatin M

Oncostatin M (OSM) is a cytokine that was initially described as a regulator of cell growth which could inhibit the growth of certain solid tumor cell lines and stimulate the proliferation of normal fibroblasts (Zarling *et al.*, 1986). The determination of its partial amino acid sequence led to the cloning of human OSM (Malik *et al.*, 1989). *In vitro* OSM shares many common activities with LIF and IL-6. For example, OSM, like LIF and IL-6, induces the differentiation of leukaemia cells and inhibits the differentiation of embryonic stem cells, as described for LIF. OSM also promotes the proliferation of Aids-related Kaposi's sarcoma cells by an autocrine and paracrine mechanism (Bruce *et al.*, 1992). Apart from these actions on non-neuronal cells, it has been shown that OSM promotes the survival of embryonic nodose and trigeminal ganglion neurons in culture (Horton *et al.*, 1996), although the function of OSM in the nervous system is in general poorly

understood.

4.1.6. Cardiotrophin-1

Cardiotrophin-1 (CT-1) is a novel member of the neuropoietic family of cytokines that was identified on the basis of its ability to induce cardiac myocyte hypertrophy using an expression cloning strategy. CT-1 is expressed in adult mouse heart, skeletal muscle, liver, lung, kidney, testis, and brain, suggesting that CT-1, like other cytokines, is a pleiotropic factor (Pennica *et al.*, 1995a). It has been demonstrated that CT-1 has multiple functions in the hematopoietic and nervous system, as well as in liver and heart. CT-1, like IL-6, LIF and OSM, inhibits the proliferation of M1 leukaemia cells, although, in contrast to IL-6, it does not promote B cell expansion. CT-1 can also inhibit the differentiation of embryonic stem cells, in a similar way to LIF, CNTF and OSM, and shares with LIF a common action in promoting the survival and proliferation of myocytes in culture (Pennica *et al.*, 1995b; Sheng *et al.* 1996). In the nervous system, CT-1 has been shown to support the *in vitro* survival of chicken ciliary, rat dopaminergic, and mouse sensory neurons, and induce changes in the transmitter phenotype of sympathetic neurons (Pennica *et al.*, 1995b; Horton, personal communication). However, the effects of CT-1 in the nervous system, like those of OSM, need to be further analysed.

4.2. RECEPTORS OF THE NEUROPOIETIC CYTOKINES

The functional redundancy of neuropoietic cytokines may be explained by the composition of their receptors. CNTF, LIF, IL-6, OSM, and CT-1 signal through multicomponent receptors that consist of a ligand-binding subunit (α) and one or more signal-transducing subunits (ß). Interestingly, some of these components are shared between different receptor complexes (for review, see Stahl and Yancopoulos, 1993).

The CNTF receptor complex is composed of three subunits, named CNTFRα, gp130 and LIFRß (for review, see Stahl and Yancopoulos, 1994). CNTF binds with low affinity to CNTF receptor alpha (CNTFRα), a specific cell surface protein anchored to the membrane via a glycosyl-phosphatidylinositol (GPI) linkage that is homologous to IL-6Rα (Davis *et al.*, 1991; for review, Davis and Yancopoulos, 1993). The complex CNTF-CNTFRα then binds to a 130 kD glycoprotein (gp130), which is a ß signal-transducing transmembrane receptor. This complex finally recruits LIF receptor ß (LIFRß), a gp130-related protein (Davis *et al.*, 1993a). This heterotrimer confers high affinity binding to CNTF and signal transduction. The cytoplasmic domains of gp130 and LIFRß do not apparently possess any enzymatic activity, however, ligand-induced heterodimerization of the two ß receptor components activates members of the Jak/Tyk family of non-receptor tyrosine kinases which are constitutively associated with both gp130 and LIFRß (Stahl *et al.*, 1994). Tyrosine phosphorylation of Jak/tyk proteins subsequently activates members of the signal transducer and activator of transcription (STAT) family of proteins which translocate to the nucleus and participate in the transcription of specific genes responsive to CNTF (Bonni *et al.*, 1993; Stahl and Yancopoulos, 1994). In contrast to the broad distribution of gp130 and LIFRß, CNTFRα, which confers ligand specificity, is found restricted mainly to the nervous system. CNTFRα mRNA is localized in all known CNTF-responsive cells, being widely expressed within neurons of the adult rat PNS and CNS (Ip *et al.*, 1993b). Its expression in non-neuronal tissues is limited to the adrenal gland and skeletal muscle. Interestingly, CNTFRα mRNA is expressed during development in several regions of the embryo with greatest levels within the nervous system. These findings contrast with the absence of CNTF mRNA expression during embryonic

development and suggest the existence of an unknown CNTF-related factor that may also act via the CNTF receptor complex (Ip *et al.*, 1993b). In support to this hypothesis, mice with targeted disruption of the CNTFRα gene, in contrast to CNTF deficient mice, die perinatally and display a severe reduction in the number of motoneurons (DeChiara *et al.*, 1995). Finally, it is important to indicate that CNTFRα can be released from the cell surface by phosphatidylinositol-specific phospholipase C, an enzyme that specifically cleaves GPI linkage. A released form of CNTFRα can act as a soluble mediator of CNTF responses and its physiological role has been confirmed by its presence in the cerebrospinal fluid and its release from muscle in response to peripheral nerve injury (Davis *et al.*, 1993b).

The LIF receptor complex is composed of two subunits. The first component, LIFRß, is a transmembrane protein homologous to gp130 that binds LIF with low affinity and is also involved in transducing the ligand signal (Gearing *et al.*, 1991). The second component of this complex is gp130 (Gearing *et al.*, 1992). The interaction between LIF and LIFRß recruits gp130 to the complex. This heterodimer receptor confers high affinity ligand binding and transduces LIF signal into the cytoplasm (Hall and Rao, 1992; Sato and Miyajima, 1994; Stahl *et al.*, 1994).

The IL-6 receptor complex is similar to the CNTF receptor. It contains an alpha binding subunit, named IL-6 receptor alpha (IL-6Rα), that is homologous to CNTFRα and binds IL-6 directly with low affinity. The second IL-6 receptor component is gp130. Monomeric gp130 homodimerizes following interaction with the IL-6/IL-6Rα complex and initiates signal transduction (Taga *et al.*, 1989; Murakami *et al.*, 1993; for review, see Kishimoto *et al.*, 1992).

OSM binds directly with low affinity to gp130. LIFRß interacts with this complex to create high affinity OSM binding (Gearing *et al.*, 1992). In contrast, CT-1, like LIF, binds first with low affinity to LIFRß and then forms a complex with gp130 that transduces the ligand signal (Pennica *et al.*, 1995b).

As in the case of the CNTF receptor, the receptor complexes for LIF, IL-6, OSM, and possibly CT-1, activate the Jak/Tyk signalling pathway upon binding with their respective ligands (Stahl *et al.*, 1994).

The importance of the apparent functional redundancy of some of the

members of these family of related proteins remains a mystery, but it could be an effective way to avoid the deleterious effect of mutations affecting a unique gene. Furthermore, the presence of receptors such as CNTFRα and IL-6Rα, that confer ligand specificity, seems an effective way to restrict the biological effects of cytokines that otherwise use signalling transducers widely expressed in the body.

5. THE FAMILY OF TGF-ß-RELATED PROTEINS AND THEIR RECEPTORS

The transforming growth factor-ß (TGF-ß) superfamily of proteins is composed of a large number of structurally related, secreted polypeptides that regulate key events during development, tissue homeostasis and repair (for reviews, see Hoffmann, 1991; Sporn and Roberts, 1992; Rosen and Thies, 1992; Wall and Hogan, 1994; Kingsley, 1994). The family contains about 30 proteins with numerous and varied functions that act on almost every cell in the body. However, the effect of most TGF-ß-related proteins on neuronal survival is limited, with the exception of two recently discovered members, glial cell line-derived neurotrophic factor (GDNF) and neurturin (NTN) (Lin *et al.*, 1993; Kotzbauer *et al.*, 1996). Most members of the TGF-ß superfamily signal through a receptor complex composed of two transmembrane receptor serine/threonine kinases (Massagué, 1996a), whereas GDNF and neurturin act via multicomponent receptors that contain a common transmembrane receptor tyrosine kinase (for review, see Mason, 1996; chapter 4 of this thesis).

5.1. MEMBERS OF THE TGF-ß FAMILY

The various members of the TGF-ß family are synthesized as biologically inactive precursor proteins. Cleavage of this precursor molecule and release of its carboxy-terminal region generates a mature biologically active protein of 110-140 amino acids. A common feature among most members of the family is the presence of seven to nine cysteine residues at the same relative position within the mature protein. Six of these cysteine residues form a compact structure of intrachain disulphide bridges known as the "cysteine knot". The active signalling form of TGF-ß family members is made up of homo- or heterodimers that are linked by a single interchain disulphide bridge and hydrophobic interactions (for review, see Kingsley, 1994). The topology of members of the TGF-ß superfamily is similar to that observed in members the nerve growth factor (NGF) and platelet-derived growth factor (PDGF). All of these factors contain the cysteine knot motif and four antiparallel ß-sheet strands (Schlunegger and Grütter, 1992; Daopin *et al.*, 1992;

McDonald and Hendrickson, 1993). Members of the TGF-ß superfamily are grouped into five subfamilies; the TGF-ß, the activin, the decapentaplegic and 60A subfamilies, the DVR group, and a group of distantly related gene products (for review, see Krieglstein *et al.*, 1995a).

The TGF-ß subfamily is composed of five highly homologous members, TGF-ß1, -ß2, -ß3, -ß4, and -ß5. TGF-ß1, the prototype of the TGF-ß superfamily, was the first member to be isolated and together with TGF-ß2 and -ß3 are the mammalian representatives of this subfamily. TGFß-4 and TGF-ß5 were cloned from chicken and Xenopus, respectively (Kingsley, 1994). The activin subfamily contains activins and inhibins that are homo- and heterodimers of two related subunits, inhibin-ßA and inhibin-ßB, and a distantly related inhibin-a chain. The decapentaplegic and 60A subfamily is formed by the drosophila decapentaplegic protein (DPP) and its mammalian homologues, bone morphogenetic proteins 2 and 4 (BMP2 and BMP4), and the drosophila 60A protein and its mammalian homologues, BMP5, BMP6 or Vg-related protein 1 (Vgr-1), BMP7 or osteogenic protein 1(OP-1), and BMP8 or osteogenic protein 2 (OP-2). The DVR group contains the DPP and 60A subfamily, and other related proteins, including Vg1, BMP3, growth differentation factor 1(GDF1), GDF3 or Vgr2, dorsalin and nodal. The Müllerian inhibiting substance (MIS), GDF-9, inhibin-a, GDNF and neurturin are distantly related proteins with less than 30% amino acid sequence identity to other members of the family (Kingsley, 1994).

TGF-ßs and related factors are involved in regulating numerous aspects of embryogenesis including basic body plan formation and organogenesis (Wall and Hogan, 1994). For example, they have been implicated in mesoderm induction, dorso-ventral patterning of the body axis, regression of the Müllerian duct in male embryos, and cartilage and bone formation. Furthermore, they can promote or inhibit growth depending on the cell type, modulate the immune function and regulate follicle-stimulating hormone (FSH) production in the pituitary gland (for reviews, see Massagué *et al.*, 1994; Hogan, 1996). Members of the TGF-ß family can also promote the survival of some populations of PNS and CNS neurons *in vitro* (Krieglstein *et al.*, 1995a). Embryonic rat spinal motoneurons are supported by TGF-ß1, when grown on a layer of astrocytes (Martinou *et al.*, 1990), and TGF-ß3 acts synergistically with FGF-2 to sustain purified chicken motoneurons (Gouin

et al., 1996). Midbrain dopaminergic neurons from rat embryos also survive in the presence of TGF-ß1, TGF-ß2 and TGF-ß3, and are protected by these factors against methyl-phenyl-pyridine (MPP+) toxicity (Poulsen *et al.*, 1994; Krieglstein *et al.*, 1995b). Whereas it has been shown that TGF-ß1 and TGF-ß2 exert a neurotrophic effect on neonatal rat dorsal root ganglion (DRG) neurons in culture, and TGF-ß1 action is synergistic with NGF (Chalazonitis *et al.*, 1992), neither TGF-ß1, -ß2, nor -ß3 promote the survival *in vitro* of embryonic chicken DRG neurons (Unsicker *et al.*, 1996). Furthermore, TGF-ßs can affect indirectly the survival of sensory neurons by regulating the expression of NGF and NT-3 in the target fields of NGF- and NT-3-dependent mouse sensory neurons (Buchman *et al.*, 1994).

5.1.1. Glial cell line-derived neurotrophic factor

Glial cell line-derived neurotrophic factor (GDNF) is a basic protein that was initially purified from the rat B49 cell line on the basis of its ability to promote the survival *in vitro* of rat midbrain dopaminergic neurons (Lin *et al.*, 1993; 1994). Based on data obtained from partial amino acid sequencing, rat GDNF cDNA and human genomic GDNF were cloned and their encoded amino acid sequences were found to be 93% identical (Lin *et al.*, 1993; 1994). Human GDNF gene was subsequently mapped to chromosome 5q12-p13.1 (Schindelhauer *et al.*, 1995).

GDNF represents the first member of a new subfamily of proteins within the TGF-ß superfamily. Accordingly, it is synthesized as a precursor of 211 amino acids that is cleaved and secreted as a polypeptide of 134 amino acids. Furthermore, the mature protein contains seven cysteine residues distributed in the same relative positions found in all members of the family. GDNF shares less than 20% homology with other TGFß superfamily members (Lin *et al.*, 1993). GDNF is a N-glycosylated protein with a molecular mass of 18 to 22 kDa. This mass is reduced to 15 kDa after deglycosylation. The biologically active form of GDNF is a disulphide-bonded homodimer with an apparent molecular mass on non-reducing SDS gels of 32 to 45 kDa (Lin *et al.*, 1993; 1994).

It has been demonstrated that GDNF is a pleiotropic factor with effects on neurons and non-neuronal cells. GDNF was first described as a potent neurotrophic factor for embryonic midbrain dopaminergic neurons; it promotes the

survival and morphological differentiation of dopaminergic neurons *in vitro* and increases their dopamine uptake (Lin *et al.*, 1993). Using an *in vivo* system, it has been shown that GDNF increases the survival and neurite outgrowth of embryonic dopaminergic neurons from fetal ventral mesencephalic tissue grafted to the anterior chamber of the eye of adult rats (Strömberg *et al.*, 1993; Johansson *et al.*, 1995). These GDNF responsive neurons originate from the substantia nigra pars compacta (Johansson *et al.*, 1995). Thus, dopaminergic neurons respond to GDNF both *in vitro* and *in vivo*. GDNF mRNA is expressed in the developing and adult striatum (caudate/putamen), the major target tissue for this kind of neurons (Strömberg *et al.*, 1993; Springer *et al.*, 1994), and GDNF is retrogradely transported by adult mesencephalic dopamine neurons of the nigrostriatal pathway (Tomac *et al.*, 1995a). This suggests that GDNF may act physiologically as a target-derived survival factor for both embryonic and adult dopaminergic neurons. However, mutant mice with targeted disruption of the GDNF gene do not display a loss of dopaminergic neurons indicating that GDNF is not essential for the development of these neurons (Sanchez *et al.*, 1996; Moore *et al.*, 1996).

Parkinson's disease (PD) is characterized by progressive degeneration of the substantia nigra dopaminergic neurons. The consequent dysfunction of the nigrostriatal pathway produces the clinical features of the disease: resting tremor, rigidity, bradykinesia, and akinesia. It is believed that GDNF may be an important therapeutic agent in the treatment of Parkinson's disease for rescuing the neurons that otherwise would die. Several studies have demonstrated a protective function of GDNF *in vivo* in PD animal models. In these models the degeneration of dopaminergic neurons has been induced by either the administration of specific neurotoxic reagents or the transection of their axons within the medial forebrain bundle. For example, the injection of GDNF into either the substantia nigra, striatum or ventricles can prevent and repair the effects of the dopaminergic neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) in mice and monkeys, leading to a functional recovery (Tomac *et al.*, 1995b; Gash *et al.*, 1996). GDNF can also reverse the effects of the catecholamine neurotoxin 6-hydroxydopamine (6-OHDA) in rats (Hoffer *et al.*, 1994; Bowenkamp *et al.*, 1995; 1996; Sauer *et al.*, 1995) and protect adult mesencephalic dopaminergic neurons from axotomy-induced degeneration (Beck *et al.*, 1995). GDNF also affects the

non-lesioned nigrostriatal system by increasing motor activity, dopamine levels, cell body size and the sprouting of dopaminergic fibers toward the injection site (Hudson *et al.*, 1995; Shults *et al.*, 1996). Thus, GDNF may be beneficial for the treatment of Parkinson's disease.

Although GDNF was initially isolated as a neurotrophic factor for midbrain dopaminergic neurons, the neurotrophic effect of this molecule on motoneurons has been extensively studied (Henderson *et al.*, 1994; Zurn *et al.*, 1996; Yan *et al.*, 1995; Oppenheim *et al.*, 1995; Li *et al.*, 1995; Houenou *et al.*, 1996). GDNF promotes the *in vitro* survival of embryonic rat motoneurons with a higher potency than other neurotrophic factors like BDNF, CNTF, and CDF-LIF (Henderson *et al.*, 1994), and induces choline acetyl-transferase (ChAT) activity and neurite outgrowth (Zurn *et al.*, 1994). GDNF is also able to rescue *in vivo* developing chicken motoneurons from programmed cell death when administered *in ovo* onto the chorio-allantoic membrane during their period of naturally ocurring cell death (Oppenheim *et al.*, 1995; Houenou *et al.*, 1996). Furthermore, it has been shown that GDNF is retrogradely transported by lumbar spinal motoneurons (Yan *et al.*, 1995) and prevents axotomy-induced motoneuron death and atrophy following the transection of the facial nerve in neonatal rats (Henderson *et al.*, 1994; Zurn *et al.*, 1996; Yan *et al.*, 1995; Oppenheim *et al.*, 1995). In adult rats, GDNF administered locally or systemically can prevent axotomy-induced decrease of ChAT in facial motoneurons (Yan *et al.*, 1995). GDNF administered locally can also prevent the massive loss of adult spinal motoneurons after avulsion of the ventral root and causes hypertrophy of the surviving motoneurons (Li *et al.*, 1995). GDNF mRNA is expressed in the limb bud and in primary cultures of embryonic myotubes and neonatal Schwann cells (Henderson *et al.*, 1994). In the embryonic limb bud, GDNF mRNA is initially transiently expressed by mesenchymal cells in a restricted region of the proximal limb bud where motor and sensory axon fascicles converge to enter the limb (Wright and Snider, 1996). Later in development, GDNF is expressed in the ventral root by fibroblast-like cells, in the peripheral nerve by Schwann cells and in a subset of muscles (Henderson *et al.*, 1994; Wright and Snider, 1996). In the chicken , GDNF mRNA is highly expressed in the muscle at E6, coinciding with the beginning of the period of naturally occurring spinal

motoneuron cell death, and drops markedly at later ages when neurons compete for limiting amounts of target-derived trophic support (chapter 2 of this thesis). Furthermore, GDNF deficient mice exhibit a reduction in the number of spinal motoneurons (Sanchez *et al.*, 1996; Moore *et al.*, 1996). This suggests that GDNF may act physiologically as a neurotrophic factor for motoneurons, and may be beneficial for the treatment of motoneuron degenerative diseases such as amyotrophic lateral sclerosis and spinal muscular atrophies.

A first attempt to test the therapeutic potential of GDNF on motoneuron diseases has been recently reported (Sagot *et al.*, 1996). In a mouse model of motoneuron degeneration (progressive motor neuropathy, pmn), GDNF, delivered by encapsulated GDNF-producing BHK cells (baby hamster kidney fibroblast), significantly reduces the loss of facial motoneurons but has no effect on myelinated fiber degeneration (Sagot *et al.*, 1996). Thus, GDNF may be useful in the treatment of motoneuron diseases if administered in conjunction with other trophic factors such as IGF-1 (for review, see Lewis *et al.*, 1993) that act on the nerve degeneration.

Other CNS neuronal populations that are supported by GDNF include noradrenergic neurons, cerebellar Purkinje cells, and basal forebrain cholinergic neurons (Arenas *et al.*, 1995; Mount *et al.*, 1995; Williams *et al.*, 1996). GDNF can prevent 6-OHDA-induced death of adult noradrenergic neurons localized in the locus coeruleus *in vivo*, and increase the level of tyrosine hydroxylase and promote neurite sprounting of the surviving neurons (Arenas *et al.*, 1995). This noradrenergic nucleus is severely impaired by neurodegenerative diseases of the brain. Furthermore, GDNF is a potent survival and differentiation factor for cultured cerebellar Purkinje cells from embryonic rat, whereas granule neurons and cerebellar glia do not appear to be targets of GDNF action (Mount *et al.*, 1995). It has been reported that GDNF can enhance the *in vitro* survival of embryonic rat basal forebrain cholinergic neurons after NGF deprivation, as assessed by ChAT staining (Ha *et al.*, 1996). Another study has shown that GDNF can prevent the atrophy and sustain the phenotype of adult rat cholinergic neurons in the basal forebrain after transection of the fimbria/fornix *in vivo* (Williams *et al.*, 1996; Lapchak *et al.*, 1996). Neurons in the medial septum and diagonal band of Broca

project to the ipsilateral hippocampus through the fimbria/fornix, and are of particular interest because they degenerate in Alzheimer's disease (Olson, 1994). Thus, GDNF may be of potential interest for the treatment of this disease.

GDNF mRNA is widely expressed in the CNS. Using *in situ* hybridization GDNF mRNA has been found in developing rat striatum, ventral limbic areas, and spinal cord (Strömberg *et al.*, 1993). Using the polymerase chain reaction (RT-PCR), GDNF mRNA has been localized in mesencephalic substantia nigra (Schaar *et al.*, 1994; Cristina *et al.*, 1995). In a more comprehensive study using *in situ* hybridization, GDNF mRNA has been found in other numerous regions of the developing rat CNS, including hippocampus, cerebral cortex, cerebellum, thalamus, cingulate cortex, substantia innominata, brain stem (locus coeruleus, motor and principal sensory trigeminal nuclei), pineal gland and dorsal horn of the spinal cord (Nosrat *et al.*, 1996). In the adult CNS, GDNF mRNA is widely expressed and has been detected by RT-PCR in striatum, hippocampus, cortex and spinal cord of both rat and human, and in the rat cerebellum (Springer *et al.*, 1994). Finally, using a sensitive RNase protection assay, GDNF mRNA has also been found in the adult rat septum, mesencephalon, medulla oblongata, colliculi, cochlear nucleus, and hypothalamus (Arenas *et al.*, 1995). The widespread pattern of GDNF mRNA expression in the embryonic and adult brain suggests that GDNF could provide trophic support to numerous populations of CNS neurons by either an autocrine, paracrine and /or target-derived mechanism.

The action of GDNF is not limited to the central nervous system. Several populations of neurons from the peripheral nervous system also respond *in vitro* and *in vivo* to GDNF (as discussed in chapter 2 of this thesis). Accordingly, homozygous GDNF-null mutant mice show a reduction in the number of neurons in the petrosal-nodose ganglion (40%), superior cervical sympathetic ganglion (35%), and dorsal root ganglion (23%) (Moore *et al.*, 1996). Furthermore, GDNF deficient mice display profond defects in the development of the kidney and the enteric nervous system (ENS) (Sanchez *et al.*, 1996; Pichel *et al.*, 1996; Moore *et al.*, 1996). Neurons from the enteric nervous system are completely absent in the wall of the lower oesophagus, stomach, and small and large intestine in GDNF knockout mice. Since GDNF mRNA expression is localized in the muscle layers of

the gastrointestinal tract, GDNF probably provides trophic support to innervating enteric neurons (Trupp *et al.*, 1995; Nosrat *et al.*, 1996). In GDNF knockout mice, enteric neurons develop and are present in the oesophagus and stomach wall at early stages of development, but most of them die during late embryonic ages (Sanchez *et al.*, 1996). This suggests that GDNF does not affect the migration of neural crest-derived precursors cells but is an essential survival factor for enteric neurons.

In the rodent kidney, GDNF mRNA expression is found in the condensing mesenchyme surrounding the tips of the ureter bud, the area of the prospective tubules (Nosrat *et al.*, 1996; Suvanto *et al.*, 1996). The ureter bud is an evagination of the Wolffian duct that penetrates into the metanephric blastema and branches repeatedly to form the collecting tubules and ureters. This process is due to a reciprocal inductive interaction between the ureteric bud and the adjacent metanephric mesenchyme. The metanephric mesenchyme induces the formation and arborization of the ureteric bud to give rise to the renal collecting system, and the ureteric bud induces the mesenchyme to differentiate into epithelial structures that form the glomeruli and proximal and distal part of the nephron (Saxen, 1987). In homozygous GDNF mutant mice, both kidneys and ureters are missing. In most of these animals the ureter buds do not develop. When they are present, they hardly penetrate into the metanephric mesenchyme and do not branch (Sanchez *et al.*, 1996; Pichel *et al.*, 1996; Moore *et al.*, 1996). Thus, GDNF is an essential inductive factor for ureter bud formation and branching during renal organogenesis.

In addition to the developing kidney, GDNF mRNA is present in numerous other peripheral organs and tissues, including lung, heart, liver, spleen, testis, ovary, adrenal, thyroid, pituitary and salivary glands, thymus, skin, whisker pad, glomus carotidus, teeth, vibrissae, tongue, inner and outer ear, nasal cavity, eye, and blood (Suter-Crazzolara and Unsicker, 1994; Trupp *et al.*, 1995; Nosrat *et al.*, 1996; chapter 2 of this thesis). The analysis of GDNF deficient mice has revealed that the above tissues apparently develop normally in the absence of GDNF, suggesting that it is not critical for their development.

5.1.2. Neurturin

Neurturin (NTN) is a novel neurotrophic factor that has been recently isolated on the basis of its ability to promote the survival of superior cervical ganglion neurons in culture. Data obtained by partial amino acid sequencing were used to isolate mouse neurturin cDNA as well as mouse and human neurturin genomic clones (Kotzbauer *et al.*, 1996). Neurturin is a member of the TGF-ß superfamily that belongs to the GDNF subfamily. It is synthesized as a precursor protein of 195 amino acids that upon cleavage yields a 100-residue mature protein. Neurturin contains the seven conserved cysteine residues found in the same relative position across the entire TGF-ß family. Sequence comparison has revealed that mature neurturin shares 42% similarity with mature GDNF and less than 20% with other members of the superfamily. Biologically active neurturin is an homodimer with a molecular mass of 25 kDa on non-reducing electrophoresis gels (Kotzbauer *et al.*, 1996).

Neurturin mRNA is expressed in both neonatal and adult mouse brain, heart, ovary and blood. It has been shown that neurturin promotes the survival *in vitro* of embryonic nodose, superior cervical and dorsal root ganglion neurons, and its action is mediated via the MAP kinase signalling pathway (Kotzbauer *et al.*, 1996).

5.2. RECEPTORS OF THE TGF-ß FAMILY OF PROTEINS

The various members of the TGF-ß superfamily signal through a complex of two distinct transmembrane receptors, termed type I and II, that constitute a unique serine/threonine kinase (STK) receptor family (for review, see Massagué *et al.*, 1994). TGF-ßs bind to the type II receptor, TßR-II, which is a constitutively active STK that does not require ligand for activation (Lin *et al.*, 1992; Ebner *et al.*, 1993; Attisano *et al.*, 1993; Franzén *et al.* 1993; Wrana *et al.*, 1994). Certain proteins act as modulators of the binding of TGFß family ligands to their signalling receptor complexes. One such protein is betaglycan, a transmembrane proteoglycan that is also known as a TGF-ß type III receptor. Betaglycan binds TGF-ßs and regulates their access to the type II receptor, either by enhancing or inhibiting the interaction (Wang *et al.*, 1991; Lin and Lodish, 1993). The complex TGF-ß/TßR-II recruits TßR-I, which can only recognize the ligand when it is bound to the type II receptor, and the type I receptor is subsequently phosphorylated by TßR-II (Wrana *et al.*, 1994). Phosphorylation of TßR-I occurs on serine and threonine residues within a highly conserved 30 amino acid region known as the "GS domain", a glycine/serine rich region located immediately upstream of the kinase domain (Wrana *et al.*, 1994). Phosphorylation allows the type I subunit, the transducer of the TGF-ß receptor complex, to propagate the ligand signal to downstream components (Wrana *et al.*, 1994; Wieser *et al.*, 1995). Binding of TGF-ß-related proteins induces serine phosphorylation and nuclear translocation of a novel transcription factor initially cloned in drosophila and termed Mother against Dpp (MAD) that has numerous homologues in several species, including human, indicating their involvement in the TGF-ß signalling cascade (Raftery *et al.*, 1995; Sekelsky *et al.*, 1995; Liu *et al.*, 1996). Different MAD protein isoforms may transduce the signalling of distinct subsets of TGF-ß ligands and generate different biological responses by activation of specific genes (Graff *et al.*, 1996; for review, see Massagué, 1996a). Thus, the TGF-ß signalling cascade represents a novel mechanism of signal transduction distinct from that known to be employed by the members of the receptor tyrosine kinase family.

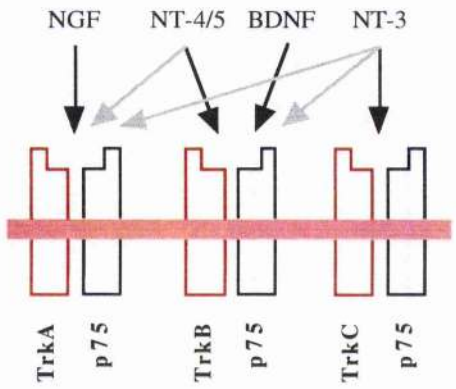
Apart from the TßR-I and TßR-II for the TGF-ß subfamily of ligands, the type I and II receptors for activin, Dpp and BMP have also been cloned and belong

to the same family of serine/threonine kinase receptors (Mathews and Vale, 1991; Mathews *et al.*, 1992; Attisano *et al.*, 1992; 1993; Nellen *et al.*, 1994; Brummel *et al.*, 1994; Penton *et al.*, 1994; Xie *et al.*, 1994; Ruberte *et al.*, 1995; Massagué, 1996a). The mechanism of activation of the activin receptor complex is similar to that described above, but the Dpp and BMP receptor complexes both contain type I and II subunits that bind directly to the ligand and are required for signal transduction (Ruberte *et al.*, 1995; Massagué, 1996a).

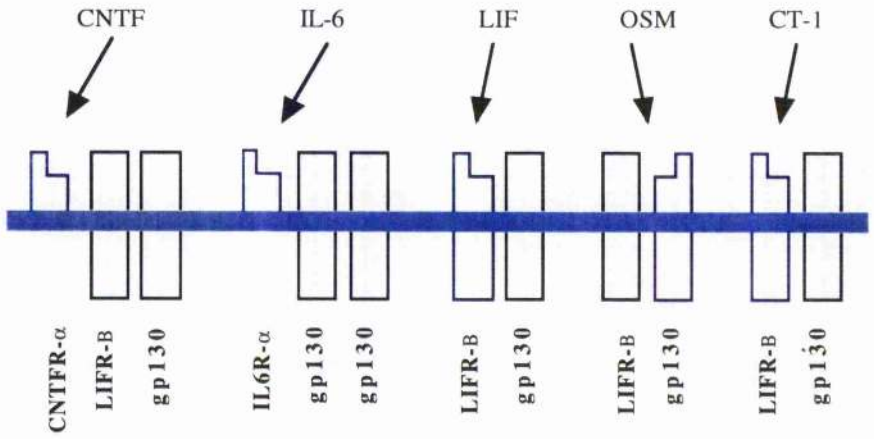
GDNF and neurturin signals are transduced into the cytoplasm by multicomponent receptors that differ from the other TGF-ß family of receptors (as described in chapter 4 of this thesis). It has been demonstrated that GDNF binds with high affinity to a glycosyl-phosphatidylinositol-linked ligand specific receptor, named GDNF receptor alpha (GDNFR-α) (Jing *et al.*, 1996; Treanor *et al.*, 1996). The current model for the GDNF receptor activation proposes that the complex GDNF/GDNFR-α associates with a signal transducer component, a transmembrane receptor tyrosine kinase termed RET (Takahashi *et al.*, 1988; 1989; Trupp *et al.*, 1996; Durbec *et al.*, 1996; Vega *et al.*, 1996; Worby *et al.*, 1996). This interaction induces an heterodimerization of the receptor components and RET is activated by autophosphorylation on tyrosine residues (for reviews, see Mason, 1996; Robertson and Mason, 1997).

The receptors for the TGF-ß superfamily of ligands described to date belong to the family of plasma membrane serine/threonine kinases. The significance of the existence of other members of the TGF-ß family that signal through receptor tyrosine kinases (RTK) remains to be clarified. It is possible that other TGF-ß-related proteins also interact with either RET or other RTKs, since it has been shown that in certain cells, TGF-ß1 and TGF-ß2 can bind to three unidentified GPI-linked cell surface proteins with molecular masses of 60 kDa, 140 kDa and 180 kDa (Massagué, 1992). Alternatively, GDNF and neurturin may belong to a new family of growth factors at the interface between the neuropoietic cytokine and TGF-ß families.

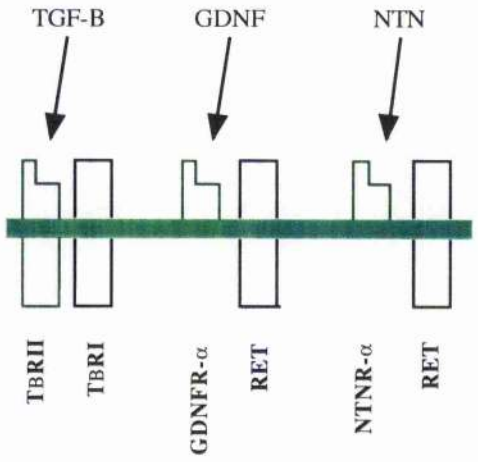
NEUROTROPHINS



CYTOKINES



TGF-B-RELATED PROTEINS



6. AIMS OF THIS PROJECT

The aims of this project were three-fold. First, to investigate the *in vitro* responsiveness of various populations of neurons from the peripheral nervous system to GDNF during embryonic development and to analyse GDNF mRNA expression in the tissues innervated by these neurons. Second, to study the survival-promoting effects of neurturin on PNS neurons in culture. Third, to isolate chicken GDNFR-α and related cDNAs, to study the function of the cloned receptors, and to determine the developmental expression of the receptor mRNAs in chicken tissues.

IX. CHAPTER 2

EXPRESSION AND NEUROTROPHIC ACTIONS OF GDNF IN THE PERIPHERAL NERVOUS SYSTEM

1. INTRODUCTION

The survival of many populations of neurons in the developing vertebrate nervous system depends on trophic support from their innervation targets and, in some cases, from the afferents they receive (Oppenheim, 1991). An extensively studied family of trophic factors are the neurotrophins: nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), NT-4/5 and NT-6. *In vitro* studies have shown that each of these factors is able to support the survival of distinct classes of neurons (Davies, 1994a). The physiological relevance of many of these *in vitro* studies has been confirmed by analysis of the phenotypes of mice with targeted null mutations in neurotrophin genes (Crowley *et al.*, 1994; Ernfors *et al.*, 1994a, 1994b; Farinas *et al.*, 1994; Jones *et al.*, 1994) and neurotrophin receptor genes (Klein *et al.*, 1993, 1994; Smeyne *et al.*, 1994). In general, populations of neurons in the peripheral nervous system that have been shown to be supported by a particular neurotrophin in culture are greatly reduced in number in mice with mutations in either the corresponding neurotrophin gene or the gene encoding its receptor tyrosine kinase (Davies, 1994c; Snider, 1994).

Several proteins that are not structurally related to the neurotrophins are also able to support the survival of particular kinds of neurons in culture. These include, ciliary neurotrophic factor (CNTF) (Sendtner *et al.*, 1994), basic fibroblast growth factor (bFGF) (Eckenstein, 1994), leukaemia inhibitory factor (LIF) (Murphy *et al.*, 1993) and glial cell line-derived neurotrophic factor (GDNF) (Lin *et al.*, 1993).

GDNF is a distantly related member of the transforming growth factor-beta family that was recently purified from the B49 glial cell line. It was initially described as a potent survival factor for midbrain dopaminergic neurons (Lin *et al.*, 1993) and motoneurons (Henderson *et al.*, 1994; Zurn *et al.*, 1994; Oppenheim *et al.*, 1995). Furthermore, GDNF is able to protect midbrain dopaminergic neurons from both 6-OHDA (Hoffer *et al.*, 1994) and MPTP toxicity (Tomac *et al.*, 1995b)

and from axotomy-induced degeneration (Beck *et al.*, 1995), and motoneurons from axotomy-induced cell death (Henderson *et al.*, 1994; Zurn *et al.*, 1994; Yan *et al.*, 1995; Oppenheim *et al.*, 1995). GDNF mRNA has been found to be expressed in developing striatum (Strömberg *et al.*, 1993; Schaar *et al.*, 1993) and limb muscle (Henderson *et al.*, 1994), target tissues for midbrain dopaminergic neurons and spinal motoneurons, respectively. Its potential effects on neurons of the peripheral nervous system have not, however, been extensively analyzed. Prior to the study presented in this chapter, it was thought that GDNF was a specific neurotrophic factor for midbrain dopaminergic neurons and motoneurons, raising the possibility of using this molecule as a therapeutic agent for the treatment of neurodegenerative diseases such as Parkinson's disease and amyotrophic lateral sclerosis.

The aim of this study was to analyse the neurotrophic actions of GDNF on avian embryonic PNS neurons throughout development and the expression of GDNF mRNA in the target fields of these neurons. Sensory and autonomic neurons of the peripheral nervous system can be easily obtained free of contaminating non-neuronal cells for *in vitro* studies (Davies, 1988a). Cranial sensory neurons are especially useful for ascertaining which classes of sensory neurons respond to a particular factor, because functionally distinct classes of sensory neurons are segregated into anatomically discrete groups (Davies, 1987b). The results show that GDNF is able to support the survival of sympathetic, parasympathetic, proprioceptive, enteroceptive and cutaneous sensory neurons of the chicken embryo at different stages of their development.

2. NEUROTROPHIC ACTIONS OF GDNF IN THE PNS

2.1. METHODS

2.1.1. Neuronal cultures

White Leghorn chicken eggs were incubated at 38°C in a forced-draft incubator until the required age. The trigeminal, nodose, ciliary and paravertebral sympathetic ganglia and the median component of the trigeminal mesencephalic nucleus (TMN) were dissected from chicken embryos of 6, 8, 10, 12 and 14 days incubation: stages 29, 34, 36, 38 and 40, respectively (Hamburger and Hamilton, 1951) (figure 2.1). The trigeminal ganglion was further dissected into its neural crest-derived dorsomedial pole (dorsomedial trigeminal ganglion, DMTG) and placode-derived ventrolateral pole (ventrolateral trigeminal ganglion, VLTG) (Davies, 1988a). The dissections were performed under sterile conditions in a laminar flow hood using tungsten needles under a stereo microscope.

The dissected ganglia were incubated in 0.1% trypsin/calcium- and magnesium-free Hank's balanced salt solution (HBSS) for 10, 12, 15, 20 and 25 minutes at 37°C for E6, E8, E10, E12 and E14 ganglia, respectively. The trypsinized tissue was washed twice in Hank's F-12 medium containing 10% heat-inactivated horse serum (HIHS) and mechanically dissociated by gentle trituration using a fired-polished Pasteur pipette.

Non-neuronal cells were removed from E8 and older neurons by differential sedimentation (as described by Davies, 1988a). The resulting neuronal suspensions (> 95% pure) and the dissociated cell suspensions from E6 ganglia (at which age differential sedimentation is not very effective in removing non-neuronal cells) were plated in 35 mm plastic tissue culture dishes (Nunc, Gibco) that had been pre-coated with poly-DL-ornithine (0.5 mg/ml, in 0.15 M borate buffer, overnight at RT) and laminin (20 μg/ml, in F-14 medium, 4 hours at 37°C) at a density of 500-2,000 neurons per dish. The cells were grown in 2 ml of Ham's F14 medium plus 10% HIHS, with or without different purified recombinant neurotrophic factors (GDNF, BDNF, NGF or CNTF), in a humidified 4% CO_2 incubator at 37°C .

Six to eight hours after plating, the number of attached neurons within a 12 x 12 mm square in the centre of each dish was counted using an inverted phase-

contrast microscope (usually 100-200 neurons). The number of surviving, bright-phase and process-bearing neurons in this same area was subsequently counted 48 hours after plating (by which time almost all neurons had degenerated in control cultures, i.e. dishes without neurotrophic factors). The number of surviving neurons at 48 hours is expressed as a percentage of the number of attached neurons at 6 to 8 hours. In each experiment, triplicate cultures were set up for all conditions.

2.2. SOLUTIONS AND MEDIA

HBSS

Hank's balanced salt solution without calcium and magnesium (GibcoBRL).

0.1% TRYPSIN

50 mg of trypsin (Worthington) was added to 5ml of Ca/Mg free PBS (GibcoBRL) and sterilised with an 0.22 μm filter (Nalgene).

POLY-DL-ORNITHINE

0.5 mg/ml poly-DL-ornithine (Sigma) in 0.15 M boric buffer (4.6 gr boric acid, BDH, in 500 ml distilled water, pH 8.4) was sterilised with an 0.22 μm filter (Gelman Sc.).

L-15

A 1L unit of L-15 powder (GibcoBRL) was added to 1 L of double distilled water plus 100 mg streptomycin (Sigma) and 60 mg penicilin (Sigma), pH 7.3, and sterilised with an 0.22 μm filter (Gelman Sc.).

HAM'S F-12

A 1L unit of F-12 powder (Gibco BRL) was added to 1 L of double distilled water plus 100 mg streptomycin (Sigma) and 60 mg penicilin (Sigma), pH 7.3, and sterilised with an 0.22 μm filter (Gelman Sc.).

HAM'S F-14

10X concentrated stock solution:

A 5L unit of F-14 powder (Imperial) was added to 500 ml of double distilled water containing 500 mg streptomycin (Sigma) and 300 mg penicilin (Sigma). The 10x stock was stored in 50 ml aliquots at -40°C.

1X solution:

A 50 ml 10X aliquot was diluted into 450 ml double distilled water. 1 g of $NaHCO_3$

(BDH) was added and the solution was adjusted to pH 7 with dry ice and sterilised with an 0.22 μm filter (Gelman Sc.).

HIHS

Heat-inactivated horse serum (Gibco).

NEUROTROPHIC FACTORS

Recombinant rat NGF was a gift from John Wislow and Gene Burdon.

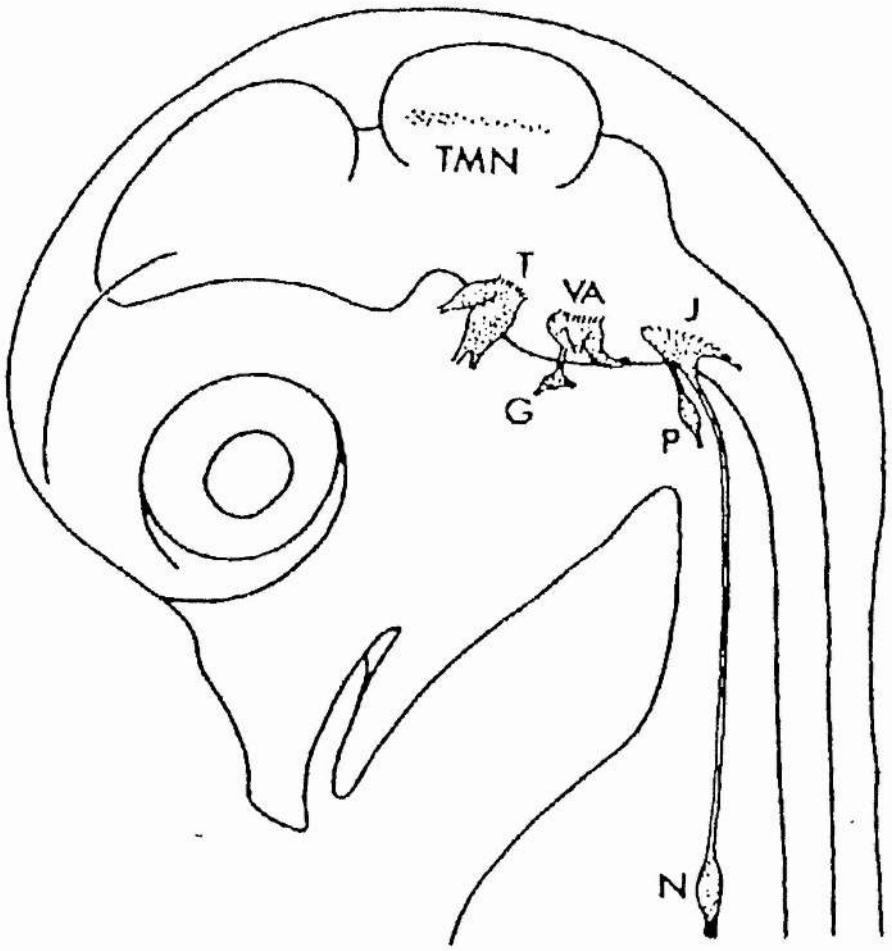
Recombinant rat BDNF was a gift from John Wislow and Gene Burdon.

Recombinant rat CNTF was a gift from David Shelton and Rae Nishi.

Recombinant rat GDNF was a gift from Arnon Rosenthal.

Neurotrophic factors were kept in F-14 plus 10% HIHS, pH 5.5, at -80°C until use.

Figure 2.1. Location of the trigeminal and nodose ganglia, and the trigeminal mesencephalic nucleus in the chicken embryo. Lateral aspect of the head of an E10 chicken embryo showing the locations of the trigeminal mesencephalic nucleus (TMN), and the trigeminal (T), geniculate (G), vestibulo-acoustic (VA), petrosal (P), and nodose ganglia (N). From Davies, 1988a.



2.3. RESULTS

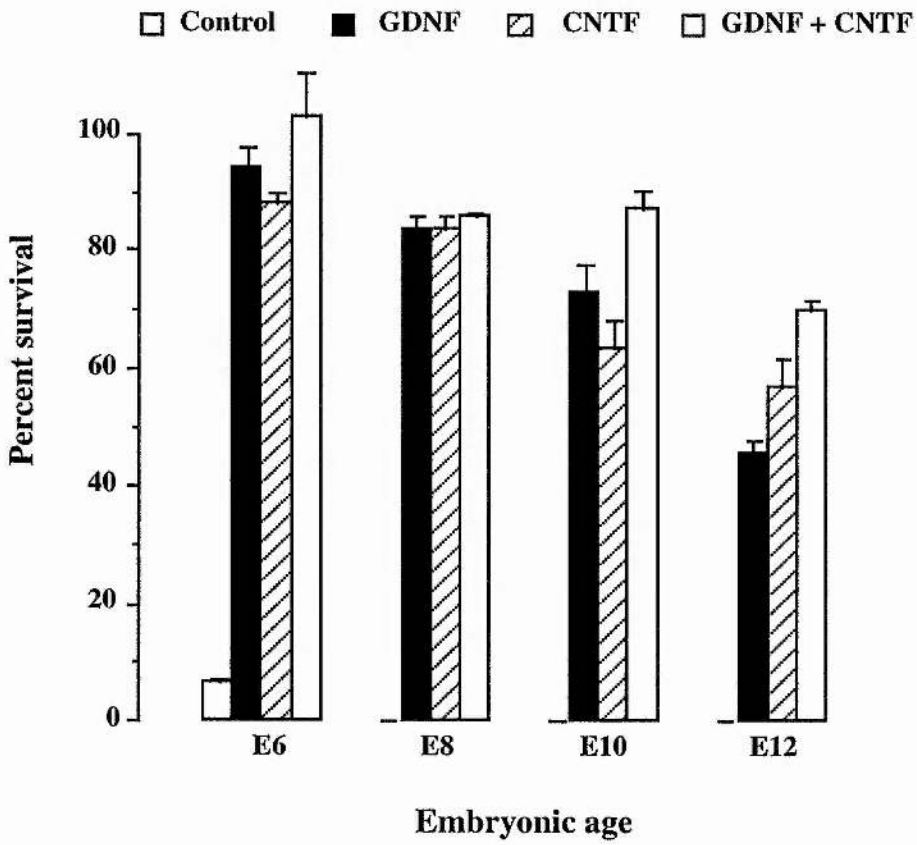
2.3.1. Parasympathetic neurons

The ciliary ganglion comprises parasympathetic neurons that innervate the iris, ciliary body and choroid and are supported by CNTF in culture (Barbin *et al.*, 1984; Buj-Bello and Davies, unpublished data) but not by neurotrophins (Allsopp *et al.*, 1993). To determine if GDNF is able to promote the survival of ciliary neurons, low-density, glial-free cultures of these neurons were established from E6, E8, E10 and E12 embryos (figure 2.2.A). After 48 hours incubation, virtually all neurons in control cultures (those not supplemented with neurotrophic factor) had died whereas the majority of neurons were surviving with GDNF. At each age, CNTF promoted the survival of similar numbers of ciliary neurons as GDNF, and there was only a small increase in the number of neurons surviving in the presence of both factors. Thus, the subsets of ciliary neurons that respond to GDNF and CNTF are largely overlapping.

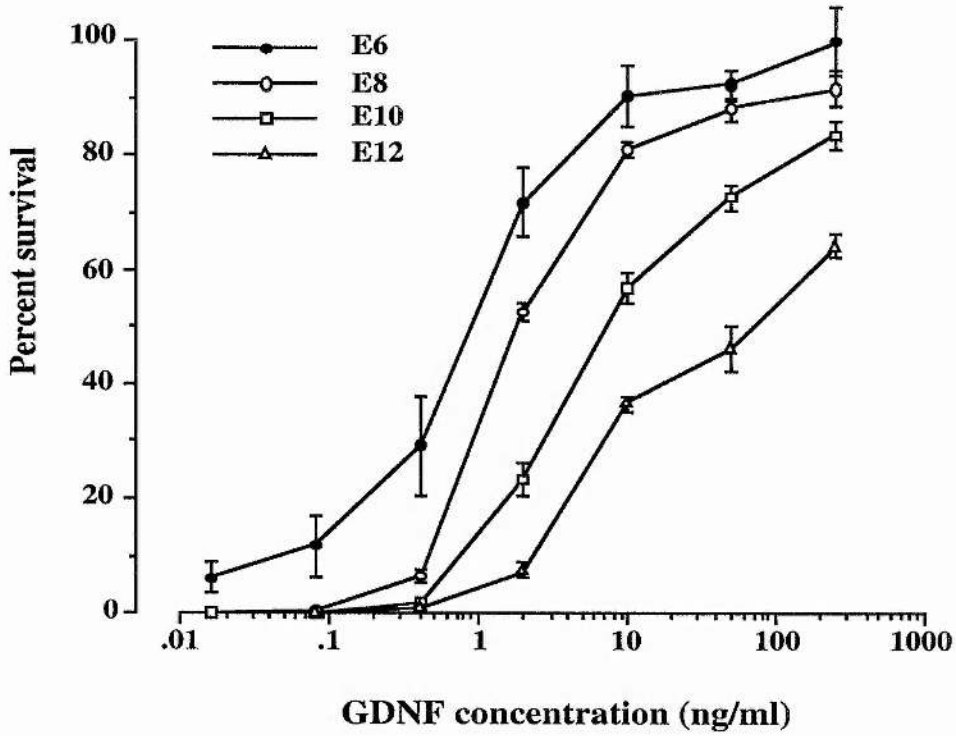
The dose-responses of ciliary neurons to GDNF shifted to higher concentrations with increasing age. There was a 10-fold increase in the half-maximally effective concentration (EC_{50}) from 0.8 ng/ml in E6 cultures to 8 ng/ml in E12 cultures (figure 2.2.B). GDNF was less potent than CNTF; the EC_{50} for E10 ciliary neurons responding to CNTF was approximately 0.05 ng/ml (data not shown).

Figure 2.2. Survival responses of E6, E8, E10 and E12 ciliary neurons to GDNF.

2.2.(A). Bar chart comparing the percent survival of ciliary neurons in control cultures and cultures containing GDNF, CNTF, and GDNF plus CNTF. The number of surviving neurons after 48 hours incubation is expressed as a percentage of the number of attached neurons counted 6 hours after plating. CNTF was used at a saturating concentration of 10 ng/ml and GDNF was used at a concentration of 50 ng/ml. The mean and standard error are shown (n = 6 for E6 and E12 cultures; n = 3 for E8 and E10 cultures).



2.2.(B). Graph of the dose-responses of ciliary neurons to GDNF. GDNF was used at concentrations ranging from 0.64 pg/ml to 250 ng/ml. The mean and standard error of the percent survival after 48 hours incubation are shown (n = 12 for E6 cultures; n = 3 for E8 and E10 cultures; n = 6 for E12 cultures).



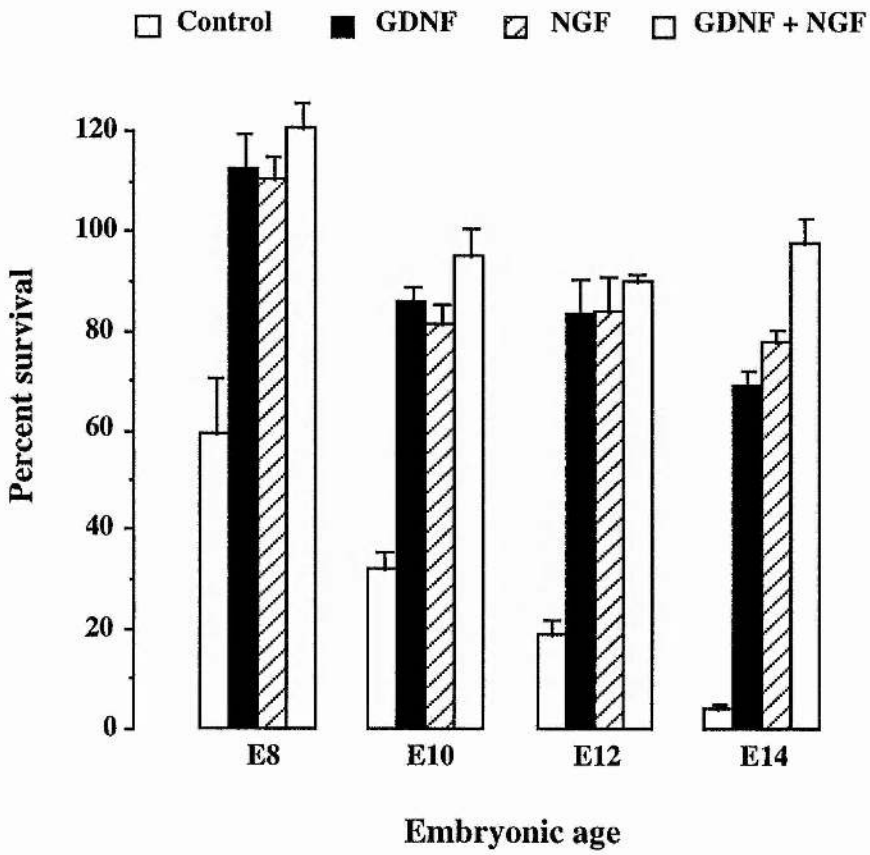
2.3.2. Sympathetic neurons

The sympathetic neurons of the embryonic paravertebral sympathetic chain are supported by NGF and CNTF in culture (Chun and Patterson, 1977; Barbin *et al.*, 1984; Buj-Bello and Davies, unpublished data). To determine if these neurons also respond to GDNF, low-density, glial-free neuronal cultures were established from E8, E10, E12 and E14 embryos. In agreement with the previous demonstration that early sympathetic neurons survive for several days in the absence of neurotrophic factors (Ernsberger *et al.*, 1989b), the number of neurons surviving in E8 control cultures was still high after 48 hours incubation (figure 2.3.A). Despite the large number of neurons surviving in control cultures, GDNF and NGF both clearly enhanced the survival of neurons at this age. The increase in the total number of neurons in E8 cultures after 48 hours incubation reflects proliferation of early sympathetic neurons (Rohrer and Thoenen, 1987; Rothman *et al.*, 1978). The number of neurons surviving in E10 and older control cultures decreased markedly. In these cultures, GDNF and NGF each promoted the survival of the majority of neurons and there was negligible additional neuronal survival in the presence of both factors (figure 2.3.A). Furthermore, like NGF, GDNF can promote the survival of sympathetic neurons at multiple embryonic ages. Thus, the subsets of sympathetic neurons that respond to GDNF and NGF are largely overlapping.

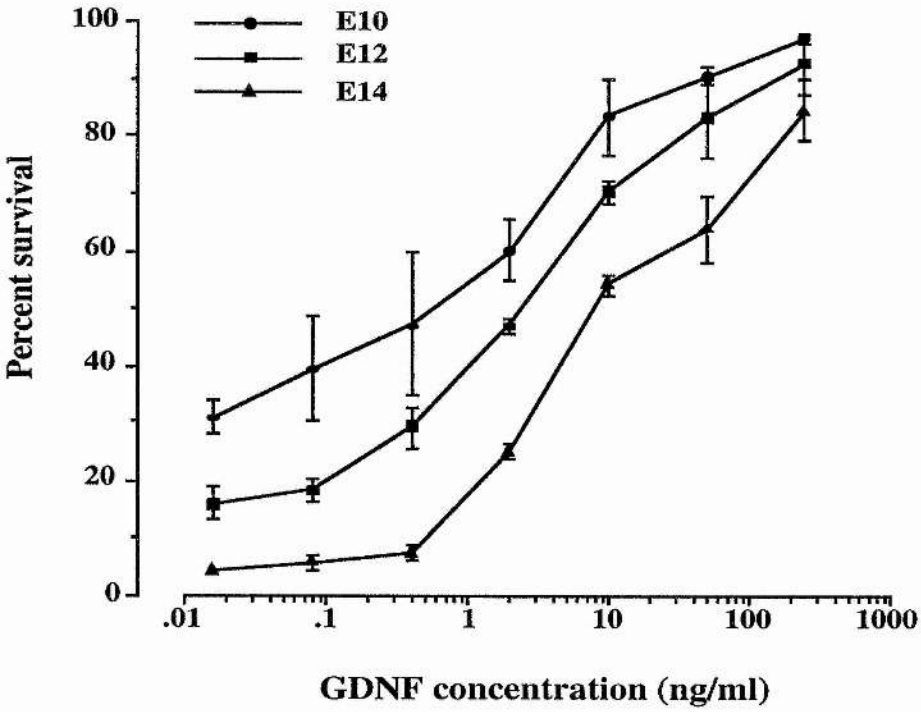
As was observed for ciliary neurons (figure 2.2.B), there was a shift in the half-maximally effective concentration (EC_{50}) of GDNF acting on sympathetic neurons. The EC_{50} increased approximately two-fold from 2.6 ng/ml at E10 to 5.7 ng/ml at E14 (figure 2.3.B). Although saturating levels of GDNF were as effective as NGF in promoting the survival of sympathetic neurons, GDNF was less potent than NGF. The EC_{50} for E12 sympathetic neurons responding to GDNF (3.4 ng/ml) was over two orders of magnitude greater than the EC_{50} for E12 sympathetic neurons responding to NGF (0.02 ng/ml) (data not shown).

Figure 2.3. Survival responses of E8, E10, E12 and E14 sympathetic neurons to GDNF.

2.3.(A). Bar chart comparing the percent survival of sympathetic neurons after 48 hours incubation in control cultures and cultures containing GDNF, NGF, and GDNF plus NGF. NGF was used at a saturating concentration of 10 ng/ml and GDNF was used at a concentration of 50 ng/ml. The mean and standard error are shown (n = 12 for E8 cultures; n = 6 for E10 and E12 cultures; n = 3 for E14 cultures).



2.3.(B). Graph of the dose-responses of sympathetic neurons to GDNF. GDNF was used at concentrations ranging from 16 pg/ml to 250 ng/ml. The mean and standard error of the percent survival after 48 hours incubation are shown (n = 6 for E10 and E12 cultures; n = 3 for E14 cultures).



2.3.3. Proprioceptive neurons

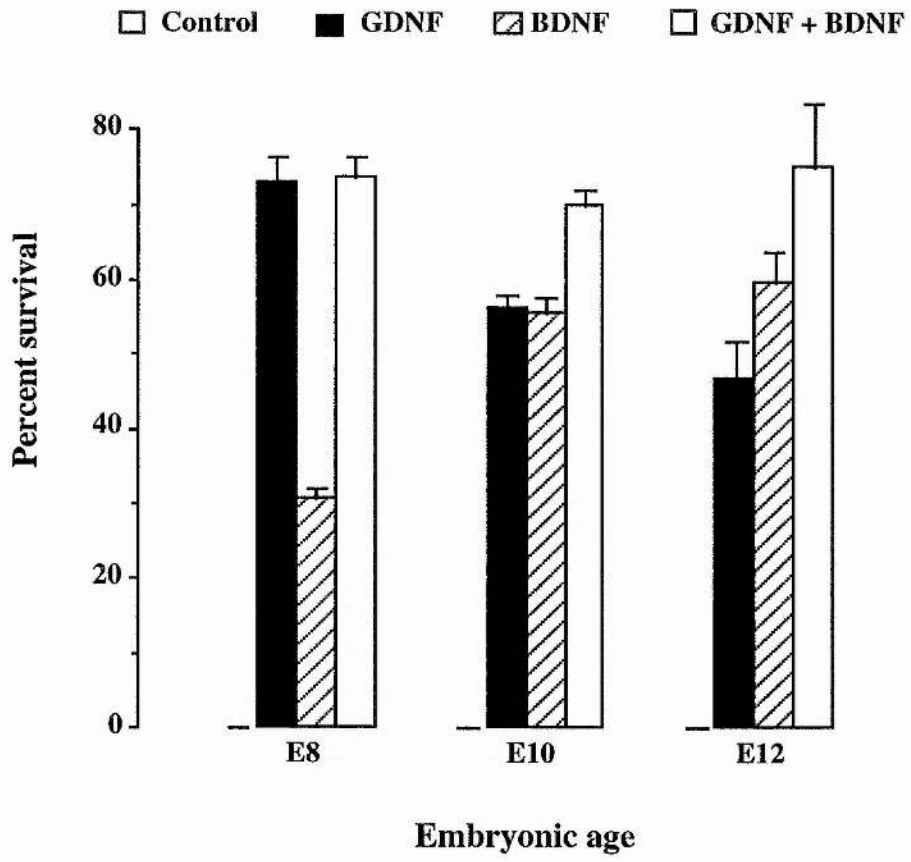
The median part of the trigeminal mesencephalic nucleus (TMN) of the chicken embryo is a circumscribed group of proprioceptive neurons that innervate stretch receptors in the jaw muscles and are supported by BDNF (Davies *et al.*, 1986b), CNTF (Buj-Bello and Davies, unplublished data) and NT3 (Hohn *et al.*, 1990) in culture. To determine if these proprioceptive neurons are responsive to GDNF, low-density, glial free cultures were established from E8, E10 and E12 embryos. In control cultures, all of the neurons died by 48 hours incubation, whereas the majority of the neurons survived with GDNF. The GDNF survival response was most marked in E8 cultures where saturating levels of GDNF promoted the survival of over 70% of the neurons. The response of TMN neurons to GDNF decreased with age, falling to about 50% in E12 cultures (figure 2.4.A).

Previous work has shown that BDNF is also an effective survival factor for cultured TMN neurons (Davies *et al.*, 1986a). However, saturating levels of BDNF were much less effective than GDNF in promoting the survival of E8 TMN neurons (30% survival with BDNF versus over 70% survival with GDNF). Similar numbers of TMN neurons were supported by BDNF and GDNF at E10 and E12. There was no additional neuronal survival in E8 cultures containing GDNF plus BDNF compared with cultures containing GDNF alone and in E10 and E12 there was a small increase in the number of neurons surviving with both factors compared with either alone (figure 2.4.A). This suggests that the populations of neurons in the TMN that respond to GDNF and BDNF are largely overlapping.

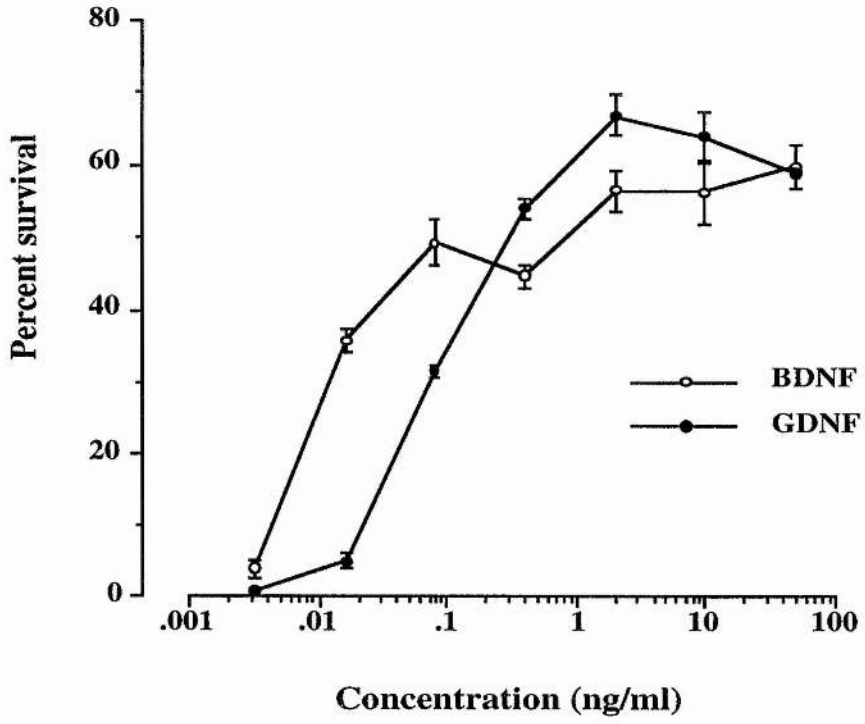
Although saturating levels of GDNF were able to promote the survival of the majority of TMN neurons in culture, GDNF was not as potent as BDNF (figure 2.4.B). The EC_{50} for E10 TMN neurons responding to GDNF (0.12 ng/ml) was an order of magnitude greater than the EC_{50} for E10 TMN neurons responding to BDNF (0.012 ng/ml).

Figure 2.4. Survival responses of TMN neurons to GDNF.

2.4.(A). Bar chart of the percent survival of E8, E10 and E12 TMN neurons after 48 hours incubation in control cultures and cultures containing GDNF, BDNF, and GDNF plus BDNF. BDNF was used at a saturating concentration of 10 ng/ml and GDNF was used at a concentration of 10 ng/ml. The mean and standard error are shown (n = 3 for E8, E10 and E12 cultures).



2.4.(B). Graph of the dose-responses of E10 TMN neurons to GDNF and BDNF. GDNF and BDNF were used at concentrations ranging from 3.2 pg/ml to 50 ng/ml. The mean and standard error of the percent survival after 48 hours incubation are shown (n = 3).



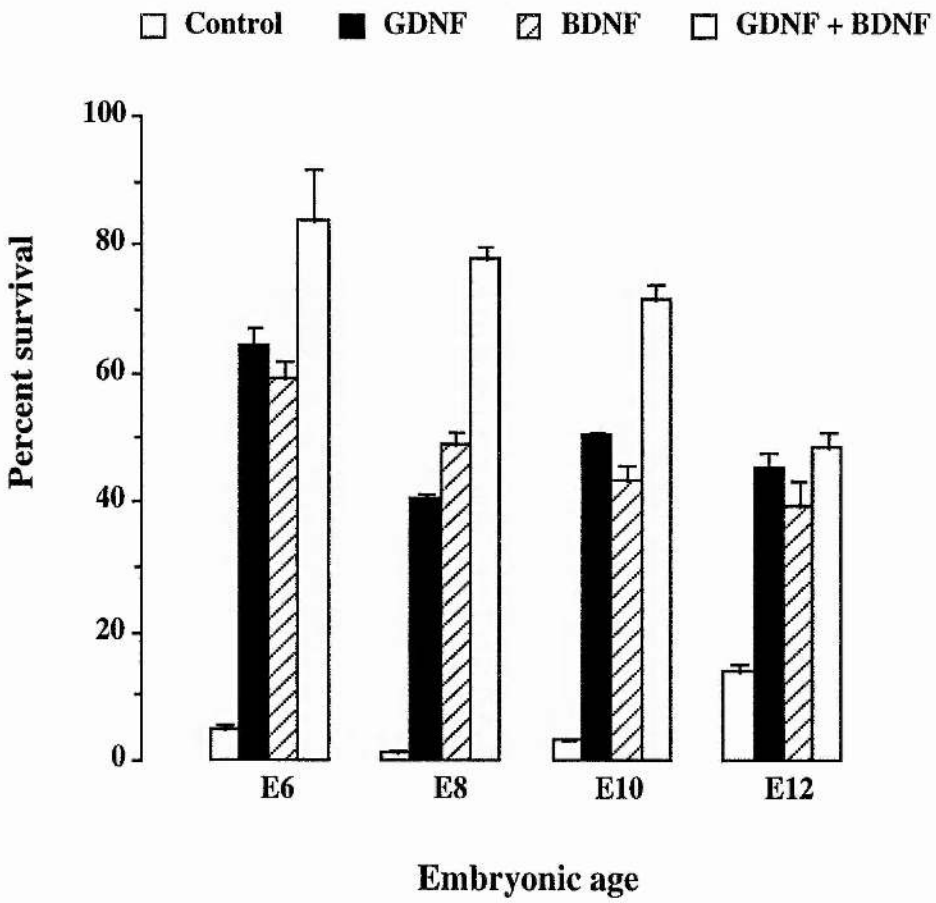
2.3.4. Enteroceptive neurons

The sensory neurons of the nodose ganglion innervate the thoracic and abdominal viscera and consist of a large subset of neurons that responds to BDNF (Lindsay *et al.*, 1985; Davies *et al.*, 1986a). To determine if nodose neurons also respond to GDNF, low-density, glial-free cultures of these neurons were established from E6, E8, E10 and E12 embryos. After 48 hours incubation most of the neurons in control cultures had died and substantial numbers were surviving with GDNF (figure 2.5.A). Between 40 and 60% of the neurons were supported by GDNF over the age range studied and similar numbers were supported by BDNF. Whereas there was no additive effect of GDNF plus BDNF on survival in E12 cultures, there was a partial additive effect of both factors at earlier ages, suggesting that the populations of nodose neurons that respond to GDNF and BDNF up to E10 are partially distinct.

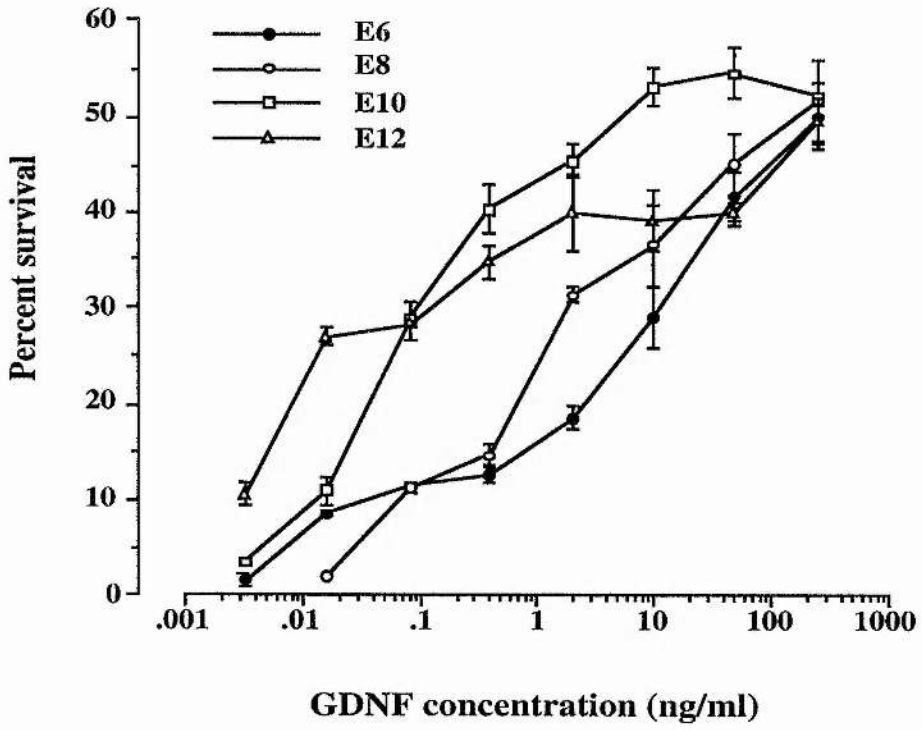
In contrast to ciliary and sympathetic neurons, which become less responsive to GDNF with increasing age, dose response studies showed that nodose neurons become more sensitive to GDNF with increasing age (figure 2.5.B). There was an approximate 50-fold decrease in the EC_{50} from 6.1 ng/ml at E6 to 0.12 ng/ml at E12.

Figure 2.5. Survival responses of E6, E8, E10 and E12 nodose neurons to GDNF.

2.5.(A). Bar chart comparing the percent survival of nodose neurons after 48 hours incubation in control cultures and cultures containing GDNF, BDNF, and GDNF plus BDNF. BDNF was used at a saturating concentration of 10 ng/ml and GDNF was used at a concentration of 50 ng/ml. The mean and standard error are shown (n = 3 for E6, E10 and E12; n = 6 for E8).



2.5.(B). Graph of the dose-responses of nodose neurons to GDNF. GDNF was used at concentrations ranging from 3.2 pg/ml to 250 ng/ml. To facilitate comparison of the EC_{50} at different ages, the E6 data were normalised to 50% survival at the highest GDNF concentration. This value is close to the percent survival for E8, E10 and E12 neurons at this concentration. The mean and standard error of the percent survival after 48 hours incubation are shown (n = 6 for E6; n = 3 for E8, E10 and E12 cultures).



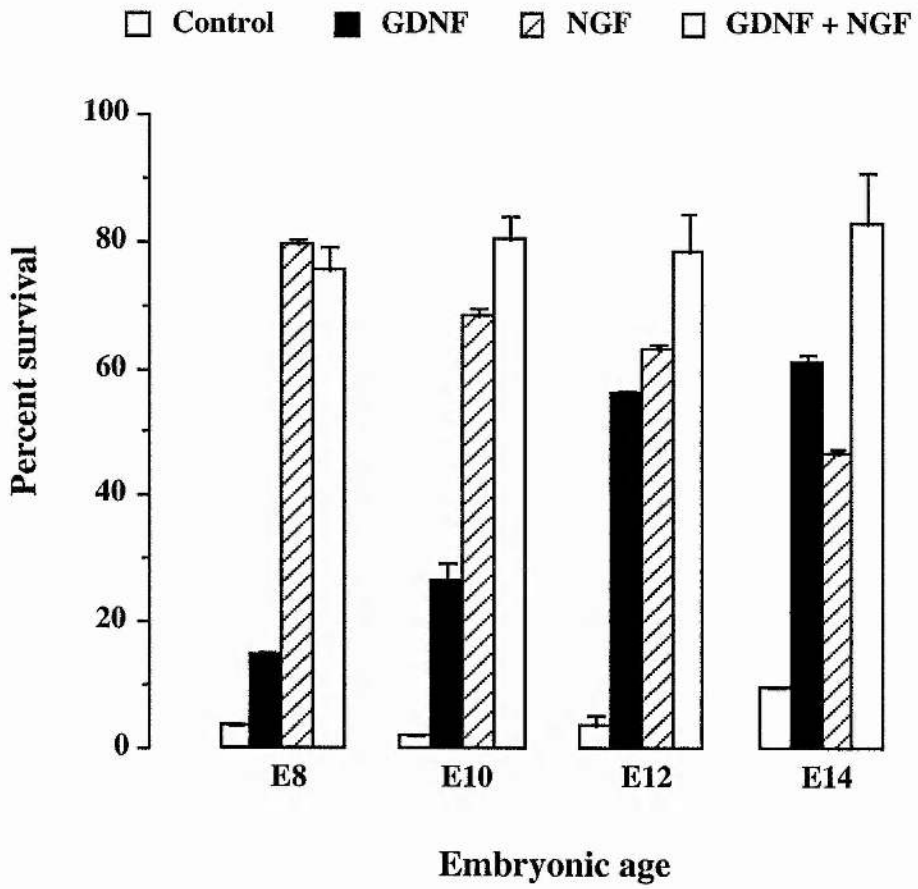
2.3.5. Cutaneous sensory neurons

The embryonic chicken trigeminal ganglion contains of two anatomically segregated populations of cutaneous sensory neurons that innervate the facial region. Those in the dorsomedial part of the ganglion (DMTG neurons) are neural crest-derived and respond to NGF, whereas those in the ventrolateral part of the ganglion (VLTG neurons) are placode-derived and respond to BDNF (Davies *et al.*, 1986a). To determine if either of these populations respond to GDNF, low-density, glial-free cultures of DMTG and VLTG neurons were established from E8, E10, E12 and E14 embryos (figures 2.6.A and 2.6.B). After 48 hours incubation virtually all of the neurons in control cultures had died, whereas the majority of DMTG neurons were supported by NGF and the majority of VLTG neurons were supported by BDNF at all ages studied.

In contrast to autonomic, proprioceptive and enteroceptive neurons, no VLTG neurons and less than 20% of DMTG neurons survived in response to GDNF at E8. There was a marked increase in the number of DMTG and VLTG neurons responding to GDNF from E10 to E12. In E12 and E14 cultures, similar numbers of DMTG neurons were supported by either GDNF or NGF (figure 2.6.A) and similar numbers of VLTG neurons were supported by either GDNF or BDNF (figure 2.6.B). A limited number of experiments showed that neither DMTG nor VLTG neurons shown any response to GDNF at E6 (data not shown). There was only a small increase in survival in DMTG cultures containing GDNF plus NGF compared with cultures containing the most effective neurotrophic factor alone at all stages studied (figure 2.6.A). In VLTG neuronal cultures, there was negligible additional survival in the presence of GDNF plus BDNF compared with cultures containing BDNF alone at all ages (figure 2.6.B). These results suggest that NGF-responsive DMTG neurons acquire GDNF responsiveness with increasing age and that BDNF-responsive VLTG neurons, likewise, acquire GDNF responsiveness as they mature.

Figure 2.6. Survival responses of E6, E8, E10 and E12 DMTG and VLTG neurons to GDNF.

2.6.(A). Bar chart of the percent survival of DMTG neurons after 48 hours incubation in control cultures and cultures containing GDNF, NGF, and GDNF plus NGF. NGF was used at a saturating concentration of 10 ng/ml and GDNF was used at a concentration of 50 ng/ml.



2.6.(B). Bar chart of the percent survival of VLTG neurons after 48 hours incubation in control cultures and cultures containing GDNF, BDNF, and GDNF plus BDNF. BDNF was used at a saturating concentration of 10 ng/ml and GDNF was used at a concentration of 50 ng/ml. The mean and standard error are shown (n = 3 for E8, E10 and E14; n = 6 for E12 cultures of both DMTG and VLTG neurons).

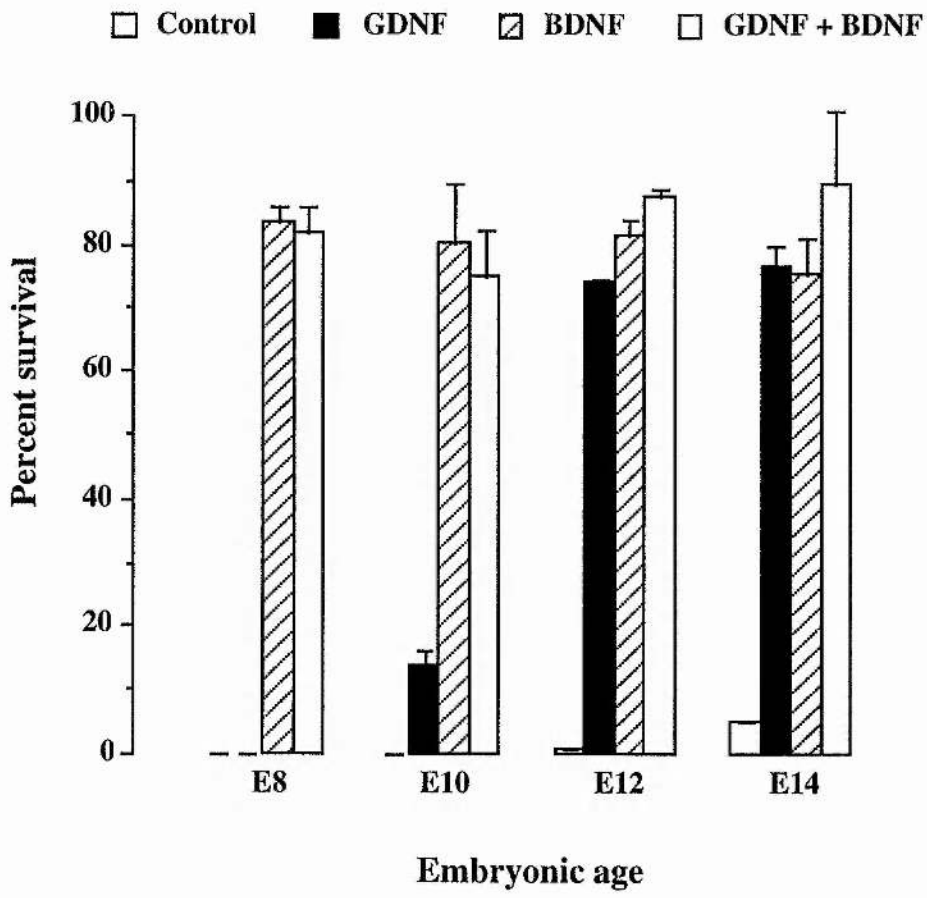
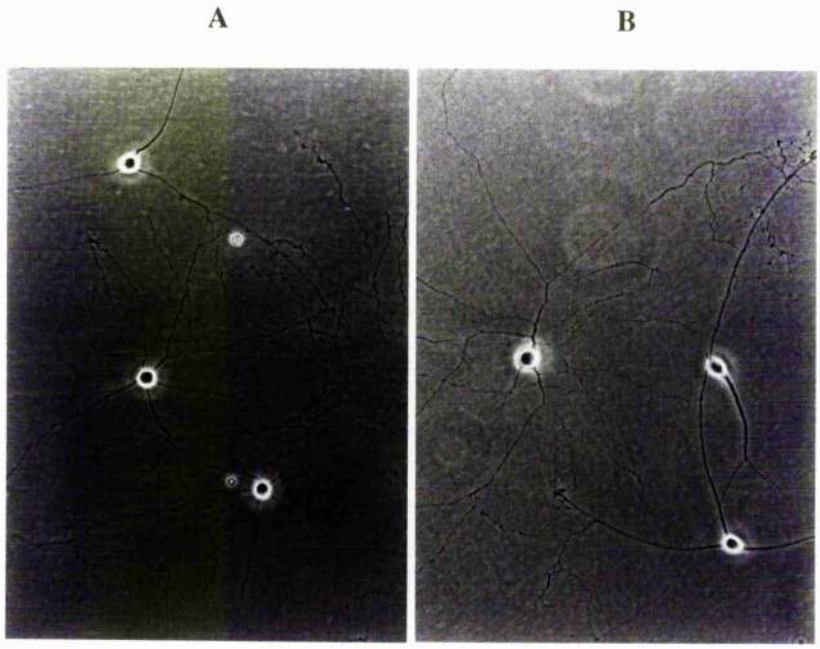


Figure 2.7. TMN neurons cultured in the presence of GDNF and BDNF.

2.7.(A). Photomicrograph of E12 TMN neurons after 48 h of incubation in cultures containing 10 ng/ml of GDNF.

2.7.(B). Photomicrograph of E12 TMN neurons after 48 h of incubation in cultures containing 10 ng/ml of BDNF.



3. EXPRESSION OF GDNF mRNA IN THE CHICKEN EMBRYO

Northern blotting was used to determine if GDNF mRNA is expressed in the tissues innervated by the autonomic and sensory neurons that respond to GDNF.

3.1. METHODS

3.1.1. Total RNA isolation

All steps were carried out at room temperature unless otherwise stated. All solutions (for composition, see Sambrook *et al.*, 1989) were exclusively used for RNA extraction, to avoid contamination with ribonucleases.

Several tissues were dissected from white leghorn chickens and rats at different embryonic and posthaching or postnatal ages. Dissected tissues were placed in polypropylene tubes and immediately frozen at -80°C until use. Total RNA was extracted using guanidinium isothiocyanate (Chizgwin *et al.*, 1979) by a single-step method (Chomczynski *et al.*, 1987).

Small amounts of tissue were usually homogenized by passing through a syringe needle in 10 µl/mg of denaturing solution (4 M guanidinium thiocyanate, 25 mM tri-sodium citrate pH 7.0, 0.5% N-lauroylsarcosine, 0.1 M 2-Mercaptoethanol). 2 M sodium acetate (1:10 vol. of the denaturing solution), pH 4, was added to the homogenate and mixed thoroughly by inversion. Water-saturated acidic phenol (1:1 vol. of the denaturing solution) was added to the tube and mixed. Chloroform (1:10 total vol.) was then added and mixed vigorously. The tubes were incubated for 15 minutes in wet ice. Then, the samples were centrifuged for 10 minutes at 15,300 rpm at 4°C. The upper aqueous phase containing total RNA was transferred to fresh tubes. The RNA was precipitated by adding 1:1 vol. of 100% isopropanol, and the tubes were placed at -20°C for periods between 30 minutes and overnight. The samples were centrifuged for 10 minutes at 15,300 rpm at 4°C, and supernatant was discarded. The pellet was washed in 70% ethanol and dried in a speed vacuum for 5-10 minutes. The RNA pellet was dissolved in denaturing solution (80 µl / 100 mg starting tissue) by vortexing. The RNA was reprecipitated with 1:10 vol. of 2 M sodium acetate, pH 4, and 2-2.5x total vol. of 100% ethanol,

and samples were placed at -20°C periods ranging between 1 h and overnight. The tubes were centrifuged for 10 minutes at 15,300 rpm at 4°C and the supernatant was discarded, and the RNA pellet was washed with 70°C ethanol and dried in a speed vacuum for 5-10 minutes. The RNA pellet was resuspended in 15 µl of MOPS buffer/100mg of starting tissue, and 2 volumes of formamide:formaldehyde (3:1) were added to the samples and mixed. The tubes were placed in a water bath at 60°C for 15-60 minutes to denature the RNA, and immediately transferred to wet ice. For gel electrophoresis, 4.5 µl of bromophenol/ethidium bromide (30/1)/100 mg of starting tissue was added to the tubes, which were stored at -80C until use.

3.1.2. Gel electrophoresis and Northern blotting

Electrophoresis of RNA was carried out in a 20x20 cm 1.2% agarose/2.2 M formaldehyde gel, using MOPS buffer, at 100-150 mA for 4-5 h. A sample of total RNA from each tube was run in an agarose/formaldehyde gel to evaluate the quality of the RNA and to calculate the concentration of RNA, according to ethidium bromide staining of ribosomal RNA and a standard RNA marker loaded on the gel. When required, the amount of RNA of each sample to be loaded on the same gel was adjusted to similar amounts.

The RNA was blotted onto Hybond-N+ filters (Amersham) overnight, and crosslinked to these membranes by a combination of UV irradiation (120,000 µjoules, Stratagene Stratalinker) and baking at 80°C for 2 hours.

3.1.3. Hybridization

For hybridization, a 400 bp probe made from the coding region of rat GDNF cDNA (plasmid obtained from A.Rosenthal) and a 437 bp probe made from the coding region of chicken cDNA glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used. The membranes were prehybridized overnight at 36°C in plastic bags containing the following solution: 50% formamide, 5 x SSC, 30 mM sodium phosphate pH 7.0, 10 mM EDTA, 0.5% SDS, 5 x Denhardt's solution and 250 µg/ml salmon sperm DNA. Filters were transferred to new bags and hybridized for 48 hours at 36°C in the above solution containing the ^{32}P-labelled nick-translated cDNA probe (Sambrook *et al.*, 1989). Following hybridization, the membranes were washed three times at 60°C, for 15 minutes, in 2 x SSC with 0.2%

SDS before exposure to Kodak Omat X-ray film.

To compare the relative levels of RNA in different lanes, the GDNF cDNA probe was stripped off the filters by boiling for 2x 5 minutes in 0.1x SSC with 0.1% SDS and the filter was subsequently hybridized with the GAPDH cDNA probe. Prehybridisation and hybridization with the GAPDH cDNA probe were carried out in a solution of the same composition as described above. Filters were prehybridized in plastic bags overnight at 42°C, and hybridized for 48 hours at 42°C in fresh solution containing the ^{32}P-labelled GAPDH probe. After hybridization, filters were washed three times at 68°C, for 15 minutes, with 2 x SSC with 0.2% SDS before being exposed to X-ray film.

3.2. RESULTS

A Northern blot of total RNA from various embryonic and postnatal tissues dissected from chickens and rats was hybridized with a ^{32}P-labelled rat GDNF cDNA probe (figure 2.8.A). Using conditions of moderate stringency, this probe hybridized to the same size transcript (0.8 kb) in RNA from rat and chicken tissues, indicating that the probe hybridizes with the putative chicken GDNF mRNA. The level of GAPDH mRNA expression was used to compare the relative levels of total RNA between each sample. Figure 2.8.A shows the presence of similar low levels of GDNF mRNA in chicken embryonic brain (E8, E12, and E16) and postnatal cerebellum (P0), hemispheres (P3), and spinal cord (P4). Lower levels of chicken GDNF mRNA was detected in muscle at P4, whereas higher levels of expression were found in postnatal gizard, liver and kidney.

GDNF mRNA was detected in all of the embryonic chicken tissues that are innervated by the classes of neurons that respond to GDNF *in vitro* including, muscle, heart, eye (figure 2.8.B), and skin (not shown). The highest levels of GDNF mRNA were found in the tissues of the eye that are innervated by ciliary neurons (choroid, ciliary body and iris). Lower levels were detected in heart (innervated by nodose neurons), limb skeletal muscle (innervated by proprioceptive neurons). Figure 2.8.B indicates that some tissues show developmental changes in the levels of expression of GDNF mRNA. In muscle the level was highest at E6 and dropped markedly at later ages. In heart the level increased from E6 to E12. In

eye tissues there was a gradual decrease in the level from E6 to E12 (figure 2.8.B). There were no obvious changes in the level of expression of GDNF mRNA in skin between E6 and E14 (not shown).

Figure 2.8.(A). Expression of GDNF mRNA in chicken and rat tissues. Autoradiogram of a Northern blot of total RNA extracted from different chicken and rat tissues hybridized with a ^{32}P-labelled nick-translated rat GDNF probe and a ^{32}P-labelled nick-translated chicken GAPDH probe.

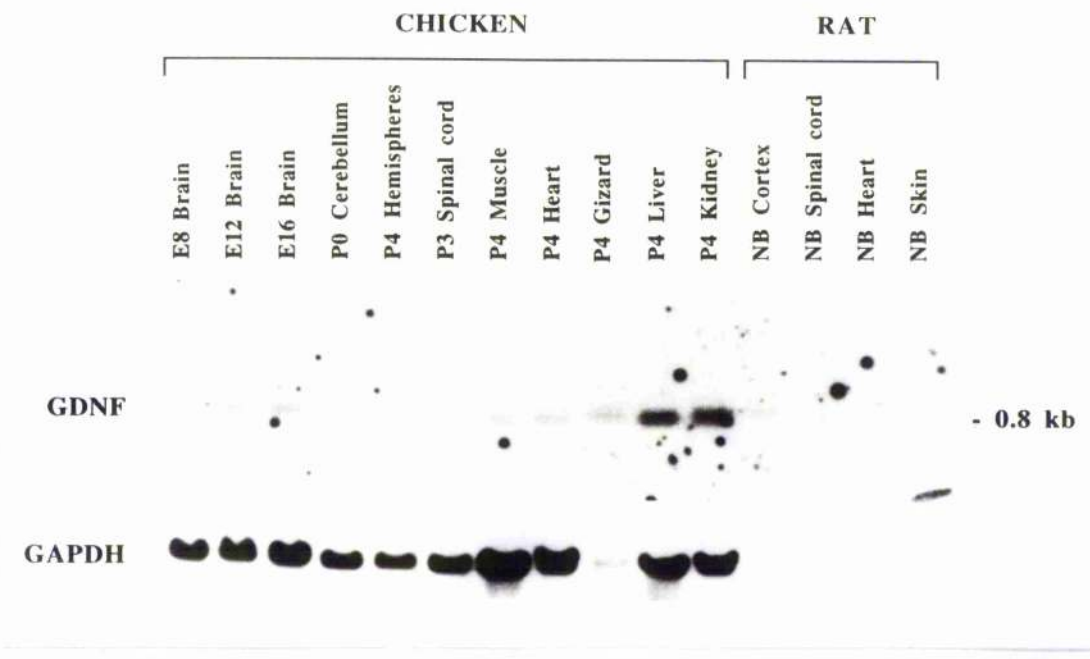
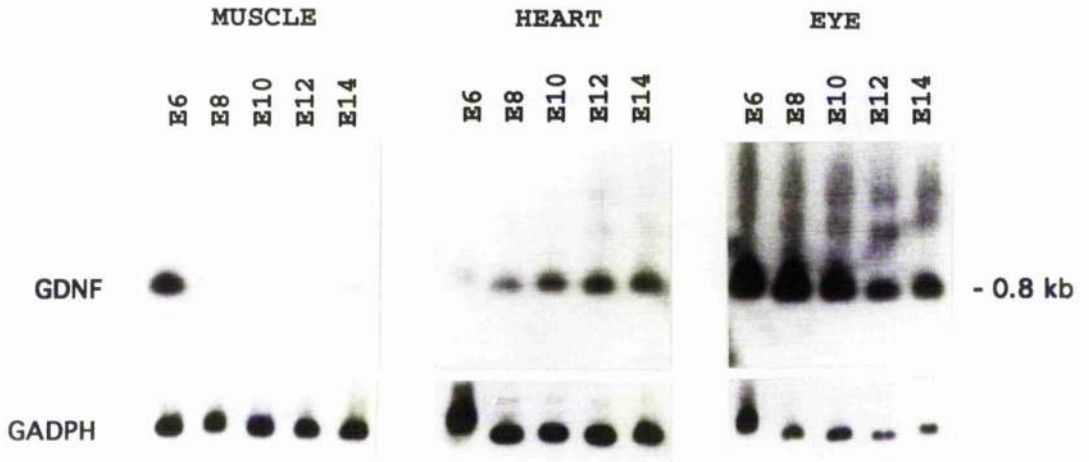


Figure 2.8.(B). Developmental changes in the expression of GDNF mRNA in tissues innervated by GDNF-responsive neurons. Autoradiograms of a Northern blot of total RNA extracted from skeletal muscle, heart and eye tissues (choroid, ciliary body and iris) of E6, E8, E10, E12 and E14 chicken embryos hybridized sequentially with a ^{32}P-labelled nick-translated GDNF probe and a ^{32}P-labelled nick-translated GADPH probe.



4. DISCUSSION

The results show that GDNF promotes the *in vitro* survival of a wide variety of neurons in the peripheral nervous system of the chicken embryo. Different populations of neurons, however, respond at different stages in their development, either becoming less sensitive or more sensitive to GDNF as development proceeds. Both sympathetic and parasympathetic neurons are supported by GDNF from an early stage of their development. With increasing age, the number of sympathetic and parasympathetic neurons supported by GDNF decreases and the neurons become less sensitive to GDNF, as shown by a shift in the GDNF dose-response to higher concentrations. Proprioceptive TMN neurons are also supported by GDNF at an early stage, and the proportion supported by GDNF falls with increasing age. Although the majority of the enteroceptive neurons of the nodose ganglion are also supported by GDNF at an early stage in their development, in contrast to autonomic neurons, these neurons become more sensitive to GDNF as they mature. In marked contrast to autonomic, proprioceptive and enteroceptive neurons, cutaneous sensory neurons do not exhibit a survival response to GDNF at an early stage in their development, but acquire responsiveness to GDNF as they mature. By E12, GDNF supports the survival of the majority of the NGF-responsive DMTG neurons and the majority of the BDNF-responsive VLTG neurons. These results suggest that GDNF plays a role in regulating the survival of various populations of PNS neurons at different stages of their development. Because each of these populations is also supported by at least one other neurotrophic factor and there is generally negligible additional survival in cultures containing this factor plus GDNF, it is likely that GDNF co-operates with various other neurotrophic factors in regulating neuronal survival.

Less comprehensive *in vitro* studies of some populations of PNS neurons of the mouse and rat embryos have revealed a limited response to GDNF (Henderson *et al.*, 1994; Trupp *et al.*, 1995). In agreement with the data presented in this chapter, E10 chicken nodose were found to be supported by GDNF in culture (Trupp *et al.*, 1995). Like in chicken, mouse nodose neurons show a survival response to GDNF. The percent survival increases from 7% survival in E15

cultures to 43% in E18 cultures, which is in line with the increasing sensitivity of developing chicken nodose neurons to GDNF with age. There is a negligible response of mouse trigeminal neurons to GDNF at either E15 or E18 (Henderson *et al.*, 1994). Similarly, E16 rat trigeminal neurons are not supported by GDNF *in vitro* (Trupp *et al.*, 1995). Given the late response of embryonic chicken dorsomedial and ventrolateral trigeminal neurons to GDNF and the more protracted development of rodent embryos compared with chicken embryos, it will be important to examine postnatal mouse trigeminal neurons to see if they too acquire GDNF responsiveness late in their maturation. Alternatively, GDNF may not play a role in the development of cutaneous sensory neurons in rodents since GDNF mRNA is present in E18 rat skin, when trigeminal neurons are unresponsive to GDNF (Trupp *et al.*, 1995), and cannot be detected in neonatal rat skin (Henderson *et al.*, 1994). These results contrast with the demonstration of GDNF mRNA in developing chicken skin and the response of the cutaneous sensory neurons of the embryonic chicken trigeminal ganglion to GDNF. Furthermore, in contrast to embryonic chicken sympathetic neurons, embryonic rodent sympathetic neurons do not respond to GDNF (Henderson *et al.*, 1994; Trupp *et al.*, 1995). It is possible that this discrepancy in GDNF responsiveness constitutes a species difference between mammals and birds. CNTF, for example, promotes the survival of embryonic chicken sympathetic neurons but is ineffective on embryonic mouse sympathetic neurons (Buj-Bello and Davies, unpublished data).

In agreement with the demonstration that GDNF promotes the *in vitro* survival of embryonic chicken sympathetic neurons, administration of GDNF to the chorio-allantoic membrane of chicken embryos results in significantly higher numbers of neurons in sympathetic ganglia compared with control embryos (Oppenheim *et al.*, 1995). However, no significant increases in ciliary, nodose or TMN neurons are observed in GDNF treated embryos (Oppenheim *et al.*, 1995). The number of dopaminergic neurons in the developing substantia nigra is also unaffected by GDNF administration in chicken embryos (Oppenheim *et al.*, 1995), yet GDNF is a potent survival factor for rat dopaminergic neurons *in vitro* (Lin *et al.*, 1993) and protects adult mouse midbrain dopaminergic neurons from both 6-OHDA (Hoffer *et al.*, 1994) and MPTP toxicity *in vivo* (Tomac *et al.*, 1995b). Although discrepancies in the effects of GDNF on avian dopaminergic neurons *in ovo* and

rodent dopaminergic neurons *in vitro* may be due to a difference in GDNF specificity in different vertebrate classes, it would be interesting to analyse the effect of GDNF on chicken dopaminergic neurons *in vitro*. However, it is unclear why GDNF does not rescue ciliary, nodose or TMN neurons *in ovo* whilst promoting the survival of these neurons *in vitro*.

The potency of rat GDNF acting on embryonic chicken PNS neurons observed in this current study is lower than that of rat GDNF acting on rat motoneurons or human GDNF acting on rat midbrain dopaminergic neurons. The EC_{50} for rat GDNF acting on rat motoneurons is 0.2 pg/ml (Henderson *et al.*, 1994) and the EC_{50} for human GDNF acting on rat dopaminergic neurons is 36 pg/ml (Lin *et al.*, 1993). In contrast, the lowest EC_{50} for rat GDNF acting on chicken neurons was 120 pg/ml for E10 TMN neurons and E12 nodose neurons. The lower potency of mammalian GDNF acting on chicken PNS neurons probably reflects cross-species differences in GDNF structure rather than lower sensitivity of PNS neurons to GDNF because rat GDNF acting on chicken motoneurons is three orders of magnitude less active than on rat motoneurons (C. Henderson, personal communication). It will be important to isolate chicken GDNF in order to ascertain its potency on various populations of chicken neurons at different stages of development.

A striking observation of this current study was the occurrence of developmental shifts in neuronal responsiveness to GDNF. Age-related shifts in neurotrophin dose responses have previously been observed in developing trigeminal ganglion neurons. For example, the sensitivity to NGF decreases by an order of magnitude to higher concentrations during the period of naturally occurring neuronal death in the embryonic mouse trigeminal ganglion (Buchman and Davies, 1993), and the loss of responsiveness of embryonic chicken DMTG neurons to BDNF and NT3 is due to shifts in the dose responses to these factors by several orders of magnitude (Buj-Bello *et al.*, 1994). In this study, it is not only demonstrated that the dose responses of some populations of neurons shift to higher GDNF concentrations with increasing age, but that the dose responses of other populations shift to lower GDNF concentrations as they mature. Because these changes in GDNF sensitivity would be expected to affect the quantities of GDNF

required for survival at different stages of development, it will be important to determine if these changes are due to differences in the expression of GDNF receptors or to intracellular signal transduction pathways.

Northern blotting has shown that an 0.8 kb GDNF transcript is expressed in all of the tissues of the embryonic chicken that are innervated by the neurons that respond to GDNF. This finding strengthens the physiological relevance of the *in vitro* results. Moreover, it is observed that developmental changes in the level of GDNF mRNA expression in some tissues seem to mirror changes in the sensitivity of the innervating neurons to GDNF. For example, the level of GDNF mRNA in heart and the response of nodose neurons to GDNF both increase during development. The level of GDNF mRNA in choroid, ciliary body and iris and the response of ciliary neurons to GDNF both decrease during development. The level of GDNF mRNA in skeletal muscle and the response of proprioceptive neurons to GDNF also decreases during development, although the decrease in GDNF mRNA is far more abrupt in muscle than in ocular tissues. If changes in the sensitivity of neurons to GDNF are related to levels of expression of the GDNF receptor, it will be important to study whether the expression of this receptor is regulated by GDNF itself. It is likely that GDNF synthesized in skeletal muscle also plays a role in supporting the survival of motoneurons which respond with increased survival to GDNF both *in vitro* (Henderson *et al.*, 1994) and *in vivo* (Oppenheim *et al.*, 1995). In skin there are no obvious changes in the level of GDNF mRNA expression over the same period of development as cutaneous sensory neurons acquire responsiveness to GDNF (not shown). It is possible that in skin GDNF may have additional functions before the innervating neurons start responding to GDNF. The synthesis of GDNF in Schwann cells (Henderson *et al.*, 1994) and the relationship of these cells to the axons of neurons that respond to GDNF raises the possibility that Schwann cells may be an additional important source of GDNF for these neurons. Type I astrocytes from the substantia nigra, basal forebrain, and cortex also express GDNF mRNA, indicating that glial cells may be a common source of GDNF for neurons (Schaar *et al.*, 1993). GDNF mRNA is expressed in various regions of the developing chicken CNS, including cerebellum, hemispheres and spinal cord. Similarly, using *in situ* hybridization, GDNF messenger has been

found in developing rat striatum (caudate/putamen), ventral limbic areas (olfactory tubercle), and Clarke's column in the spinal cord (Strömberg *et al.*, 1993). Additionally, GDNF has been shown by polymerase chain reaction (RT-PCR) to be present in mesencephalic substantia nigra (Schaar *et al.*, 1994). In the adult CNS, GDNF mRNA is widely expressed and can be detected by RT-PCR in striatum, hippocampus, cortex and spinal cord of both rat and human, and in the rat cerebellum (Springer *et al.*, 1994). These data suggest the possibility that GDNF may be a neurotrophic factor for several neuronal populations in the central nervous system, including mesencephalic dopaminergic neurons, locus coeruleus neurons, basal forebrain cholinergic neurons, cerebellar Purkinje cells and motoneurons (Lin *et al.*, 1993; Arenas *et al.*, 1995; Mount *et al.*, 1995; Williams *et al.*, 1996; Henderson *et al.*, 1994).

GDNF-null mutant mice have been generated by using homologous recombination in embryonic stem cells (Sanchez *et al.*, 1996; Pichel *et al.*, 1996; Moore *et al.*, 1996). In agreement with the results presented in this chapter on the survival-promoting effect of GDNF on PNS neurons, homozygous mice show a reduction in the number of neurons in the petrosal-nodose ganglion (40%), superior cervical sympathetic ganglion (35%), and dorsal root ganglion (23%), although no differences are detected in trigeminal sensory and vestibular ganglia in comparison to wild-type mice (Moore *et al.*, 1996). In addition, there is a significant loss of motoneurons in the lumbar spinal cord (20-30%) and trigeminal motor nucleus (20%) (Sanchez *et al.*, 1996; Moore *et al.*, 1996). However, no defects are observed in the number of midbrain dopaminergic neurons, noradrenergic neurons within the locus coeruleus, and facial motoneurons. No other deficits were detected in other regions of the CNS (Sanchez *et al.*, 1996; Pichel *et al.*, 1996; Moore *et al.*, 1996). These observations indicate that GDNF is involved in the development of several populations of PNS and CNS neurons *in vivo* and may co-operate with other trophic factors in keeping them alive. Discrepancies between the survival effects of GDNF on certain kind of neurons *in vitro*, such as dopaminergic and noradrenergic neurons (Lin *et al.*, 1993; Arenas *et al.*, 1995), and the lack of effect of knocking out the GDNF gene on these neurons indicate that GDNF is not an essential physiological survival factor for these neuronal populations during their development.

In summary, it has been demonstrated that GDNF is a survival factor for multiple classes of sensory and autonomic neurons, and that its survival promoting effects are dependent on developmental age. These results have changed the notion that GDNF is a neurotrophic factor with a highly restricted neuronal specificity.

X. CHAPTER 3

NEUROTROPHIC ACTIONS OF NEURTURIN IN THE PERIPHERAL NERVOUS SYSTEM

1. INTRODUCTION

Neurotrophic factors are molecules involved in the survival and differentiation of neurons during the development of the nervous system (Davies, 1988b). Since the discovery of NGF 40 years ago (for review, see Levi-Montalcini, 1987), the list of known neurotrophic factors is constantly increasing. Some of these factors have been isolated on the basis of their ability to promote the survival of embryonic neurons. This is the case for nerve-growth factor (NGF) (Levi-Montalcini and Hamburger, 1951), brain-derived neurotrophic factor (BDNF) (Barde *et al.*, 1982; Leibrock *et al.*, 1989), ciliary neurotrophic factor (CNTF) (Adler *et al.*, 1979; Lin *et al.*, 1989; Stöckli *et al.*, 1989), and glial cell line-derived neurotrophic factor (GDNF) (Lin *et al.*, 1993; Lin *et al.*, 1994). By the end of my Ph.D., the purification and cloning of a new neurotrophic factor, termed neurturin, was reported (Kotzbauer *et al.*, 1996). Neurturin is a 25 kDa protein that has been purified from the conditioned medium of the Chinese hamster ovary cell line on the basis of its ability to support the survival of neonatal rat sympathetic neurons. Mouse and human neurturin have been cloned and sequenced, and share about 42% identity with GDNF at the amino acid level. Neurturin is, like GDNF, a distant member of the transforming growth factor (TGF)-ß superfamily of proteins with less than 20% sequence similarity with other family members, but containing the seven conserved cysteine residues at the same relative spacing found in the entire family. Thus, GDNF and neurturin are homologous proteins that define a new subfamily of neurotrophic factors within the TGF-ß family of proteins.

The TGF-ß superfamily is composed of about 30 members that are divided in different subfamilies depending on their amino acid sequence homology; it includes the TGF-ß, the activin, and the decapentaplegic and 60A subfamilies, the DVR group, and other ungrouped proteins like Müllerian inhibiting substance (MIS) (for review, see Kingsley, 1994). They exert a wide variety of effects on cell proliferation, differentiation, and organization (for review, see Roberts and Sporn,

1990). In some cases, most members of the same subfamily, i.e. TGF-ß subfamily, have similar functions in different systems (Massagué, 1990).

In chapter 2 of this thesis it has been shown that GDNF is a survival factor for multiple classes of sensory and autonomic neurons from the chicken embryo, and that its survival-promoting action and expression in the tissues innervated by these neurons are dependent on developmental age. The physiological relevance of these *in vitro* studies has been confirmed by analysis of the phenotype of transgenic mice with a targeted null mutation of the GDNF gene (Moore *et al.*, 1996). Survival assays have shown that neurturin and GDNF both promote the *in vitro* survival of neurons from rat superior cervical ganglion (SCG), dorsal root ganglia (DRG), and nodose ganglion (Kotzbauer *et al.*, 1996). Thus, to compare further the similarities and differences between the activity of neurturin and GDNF in promoting the *in vitro* survival of chicken PNS neurons, a limited number of experiments have been performed. It has been found that neurturin, like GDNF, promotes the survival of developing sensory, enteroceptive, sympathetic and parasympathetic neurons in culture.

2. METHODS

2.1. Neuronal cultures

Nodose, ciliary and paravertebral sympathetic ganglia were dissected from E10 chicken embryos, and dorsomedial trigeminal ganglia were obtained from E14 embryos. The dissected tissue was tripsinized, washed and triturated as described in chapter 2. The neurons were separated from non-neuronal cells by differential sedimentation and plated in 35 mm dishes using culture procedures that have been also described in chapter 2. The number of surviving neurons was counted 48 hours after plating and expressed as a percentage of the number of attached neurons at 6-12 hours after plating.

Recombinant human neurturin was a gift from Arnon Rosenthal.

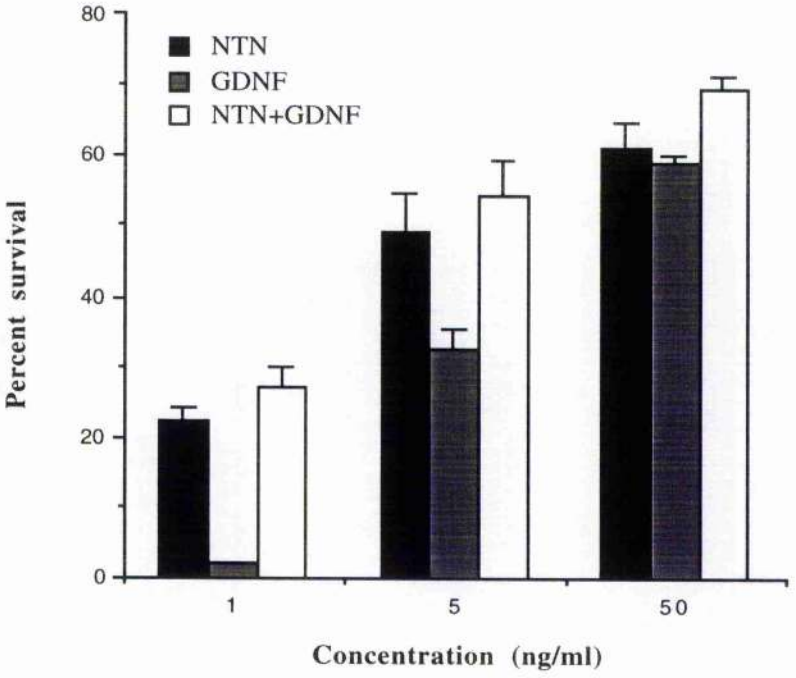
3. RESULTS

3.1. Parasympathetic neurons

The parasympathetic neurons of the ciliary ganglion are supported by CNTF / GPA (Barbin *et al.*, 1984; Eckenstein *et al.*, 1990; Buj-Bello and Davies, unpublished data) and GDNF (chapter 2 of this thesis) in culture. Glial-free cultures of E10 ciliary neurons were set up to determine whether neurturin is able to promote the survival of these neurons. After 48 hours in culture all neurons grown without neurotrophic factors had died (not shown), whereas the majority of neurons survived in the presence of high concentrations of neurturin (50ng/ml). The number of neurons surviving was similar in cultures treated with 50 ng/ml of either neurturin or GDNF, and there was only a small increase of neuronal cells surviving in the presence of both factors (figure 3.1). This indicates that the populations of ciliary neurons that respond to neurturin and GDNF are largely overlapping.

When ciliary neurons were cultured with lower amounts of either neurturin or GDNF, differences in the survival effect of these two factors were observed. There was a negligible neuronal survival in cultures with 1 ng/ml of GDNF, whereas with the same concentration of neurturin about 20% of the neurons survived. The survival of ciliary neurons was also higher in cultures treated with 5 ng/ml of neurturin than in those containing the same amount of GDNF (figure 3.1). Thus, although E10 ciliary neurons respond to both factors, neurturin is more potent than GDNF in promoting the *in vitro* survival of parasympathetic neurons.

Figure 3.1. Bar chart comparing the percent survival of E10 chicken ciliary ganglion neurons grown with neurturin, GDNF and neurturin plus GDNF for 48h. The mean and standard errors are shown (n=3 for 1ng/ml and 5 ng/ml; n=6 for 50 ng/ml).

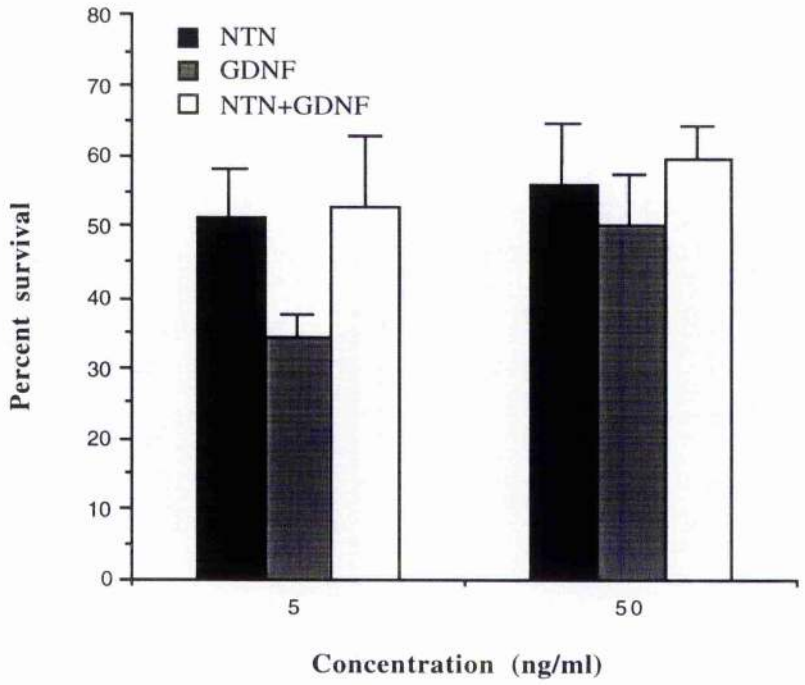


3.2. Sensory cutaneous neurons

E14 chicken DMTG neurons respond *in vitro* to NGF and GDNF (chapter 2 of this thesis), and to CNTF / GPA (Buj- Bello and Davies, unpublished data). After 48 hours of incubation, a similar number of neurons survived (about 50%) when grown with high concentrations of either neurturin or GDNF (50 ng/ml) (figure 3.2). The number of neurons alived in control dishes, i.e. without neurotrophic factors, was negligible (5%) (not shown). There was only a small increase in survival when cultures were treated with neurturin plus GDNF compared to cultures containing neurturin alone, indicating that neurturin and GDNF act on the same subpopulation of DMTG neurons. Furthermore, like GDNF, neurturin was unable to support the *in vitro* survival of E6 DMTG neurons (not shown).

However, differences in the survival-promoting activity of neurturin and GDNF were observed when E14 DMTG neurons were treated with lower amounts of these neurotrophic factors. At the same concentration (5 ng/ml) GDNF supported less neurons than neurturin (figure 3.2). Thus, like parasympathetic ciliary neurons, small-cutaneous sensory neurons are responsive to neurturin *in vitro* and are more sensitive to this factor than GDNF.

Figure 3.2. Bar chart of the percent survival of E14 chicken DMTG neurons after 48h of incubation in cultures containing neurturin, GDNF, and neurturin plus GDNF. The mean and standard error are shown (n=3).

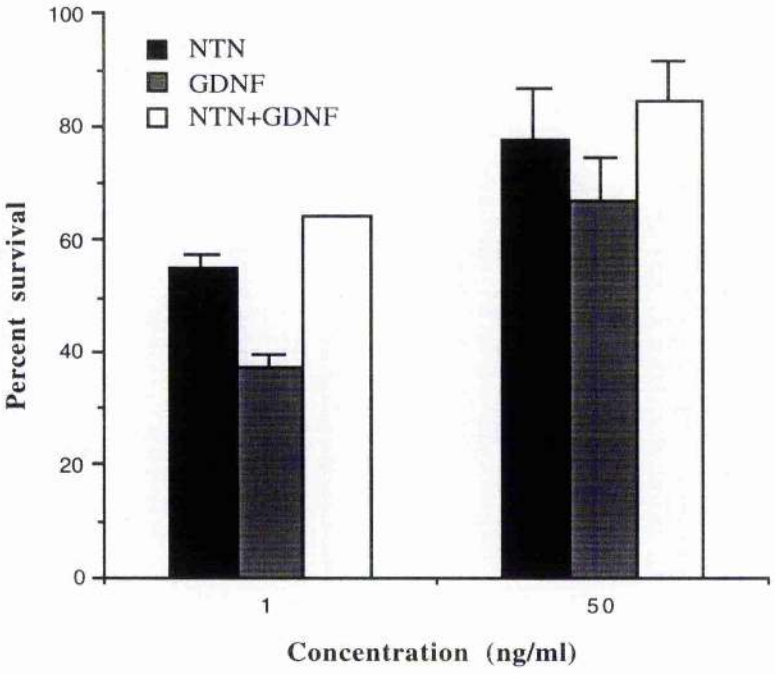


3.3. Sympathetic neurons

The chicken paravertebral sympathetic chain contains neurons that are supported *in vitro* by NGF and GDNF (chapter 2 of this thesis), and CNTF / GPA (Eckenstein et al., 1990; Buj-Bello and Davies, unpublished data). To determine whether these neurons also respond to neurturin, low-density neuronal cultures were established from E10 embryos. The number of neurons surviving in absence of neurotrophic factors after 48h was relatively high (16%) as mentioned in chapter 2 (not shown). After 48 hours of incubation, the majority of neurons survived in cultures containing neurturin at high concentrations (50 ng/ml) and there was only a small increase in the number of alive neurons when grown in combination with GDNF (figure 3.3). This indicates that the population of sympathetic neurons that respond to neurturin and GDNF is largely overlapping.

As observed for ciliary and DMTG neurons (figures 3.1 and 3.2), the number of neurons surviving in the presence of lower concentrations of neurturin (1ng/ml) was less than that observed with the same amount of GDNF, although in the case of sympathetic neurons, the difference was smaller. These results suggest that neurturin is more potent than GDNF in promoting the survival of chicken sympathetic neurons.

Figure 3.3. Bar chart showing the survival of E10 chicken lumbar chain sympathetic neurons in culture after 48h of incubation in the presence of neurturin, GDNF, and neurturin plus GDNF. The mean and standard errors are shown (n=3).

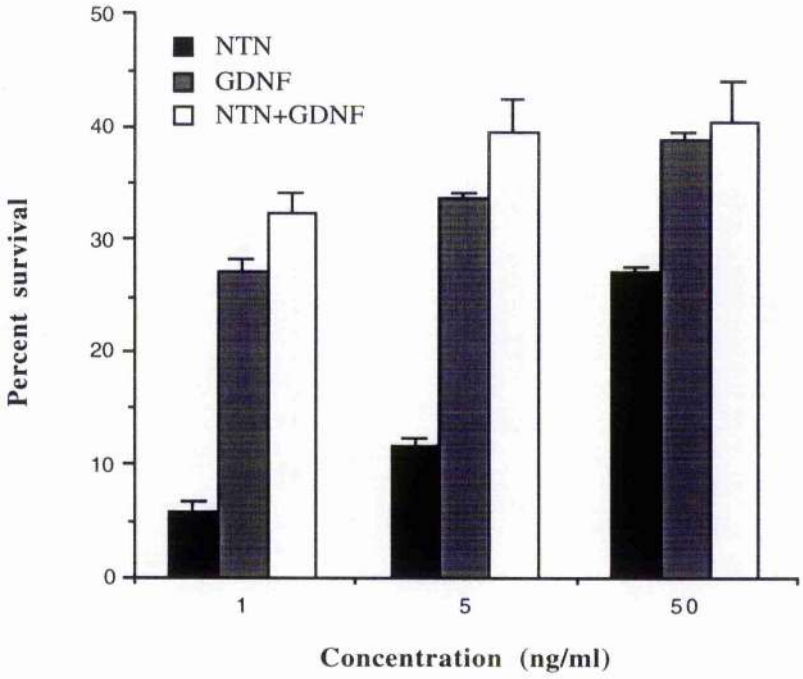


3.4. Enteroceptive neurons

The chicken nodose ganglion is composed of enteroceptive neurons that respond *in vitro* to BDNF (Lindsay *et al.*, 1985; Davies *et al.*, 1986a), NT3 (Buj-Bello *et al.*, 1994), CNTF / GPA (Buj-Bello and Davies, unpublished data), and GDNF (chapter 2 of this thesis). To assess if these neurons respond also to neurturin, E10 nodose neurons were culltured in the presence of different concentrations of either neurturin or GDNF alone and in combination. After 48 hours of incubation, high concentrations of neurturin (50 ng/ml) supported the survival of about 50% of the neurons in culture (figure 3.4), whereas negligible survival was detected in control dishes, i.e. without neurotrophic factors (not shown). Approximately the same number of neuronal cells survived with 50 ng/ml of GDNF, and there was a negligible increase in the number of surviving neurons when neurturin was added to the culture medium. Thus, the subsets of nodose neurons that respond to neurturin and GDNF are largely overlapping.

When enteroceptive neurons were cultivated with lower concentrations of either neurturin or GDNF, differences in the biological activity of these factors were observed. The number of surviving neurons at 48 hours in the presence of 5 ng/ml of neurturin was three times lower (10%) than with the same amount of GDNF (30%), and negligible at 1 ng/ml of neurturin (figure 3.4). This indicates that, in contrast to ciliary, DMTG and sympathetic neurons, nodose neurons are less sensitive to neurturin than to GDNF.

Figure 3.4. Bar chart of E10 chicken nodose ganglion neurons cultures grown for 48h with neurturin, GDNF and neurturin plus GDNF. The mean and standard error are shown (n=3 for 1ng/ml and 5 ng/ml; n=6 for 50 ng/ml).



4. DISCUSSION

The aim of this study was to determine if neurturin exerts a trophic activity on chicken PNS neurons that have previously been shown to respond to GDNF in culture (chapter 2 of this thesis). The results show that neurturin, like GDNF, promotes the *in vitro* survival of several populations of neurons from the peripheral nervous system. Sympathetic, parasympathetic, and small-cutaneous sensory neurons are more responsive to neurturin than GDNF at E10, whereas enteroceptive neurons are more sensitive to GDNF than neurturin at the same age. Using high concentrations of neurotrophic factor, the survival of sympathetic lumbar chain, ciliary ganglion and DMTG neurons is similar in response to either neurturin or GDNF, whereas nodose ganglion neurons are still more responsive to GDNF. Furthermore, the subsets of neurons within each ganglion that are supported by neurturin and GDNF are overlapping, since no additive effects were observed in cultures that contained both factors. These results suggest that neurturin may play an important role in regulating neuronal survival of several populations in the PNS during development, and it is likely that it co-operates with other neurotrophic factors, such as GDNF or neurotrophins, in keeping neurons alive.

In agreement with these results in chicken embryos, E15 dorsal root, E18 nodose, and E21 superior cervical ganglion neurons from rat embryos have been shown to be supported *in vitro* by neurturin (Kotzbauer *et al.*, 1996). Rat neurons from these ganglia were all more responsive to neurturin than GDNF in culture. In contrast, whereas in chicken embryos most of these neurons are also more sensitive to neurturin, enteroceptive neurons are less responsive to neurturin than GDNF. These discrepancies may be due to species differencies in neuronal responsiveness to these factors.

The receptors for GDNF and neurturin have been recently described. They are composed of a common transmembrane transducing receptor, termed RET (Trupp *et al.*, 1996; Durbec *et al.*, 1996; Vega *et al.*, 1996; Worby *et al.*, 1996), and a GPI-linked ligand specific receptor, named GDNFR-α (Jing *et al.*, 1996; Treanor *et al.*, 1996; chapter 4 of this thesis) and NTNR-α or TrnR-2 (Klein *et al.*, 1997; chapter 4 of this thesis; Baloh *et al.*, 1997). Similar levels of neuronal

survival are observed when cultures are treated with high concentrations of either neurturin or GDNF. This may indicate that the receptors of both factors are present in the same subpopulations of neurons in each ganglion. Alternatively, it may be due to cross-reactivity of the ligands to the receptors. Different levels of receptor expression may explain the different potency of these neurotrophic factors. It has been demostrated that GDNFR-α and NTNR-α can cross-react with either neurturin or GDNF when the ligand is present at high concentrations (chapter 4 of this thesis; Klein *et al.*, 1997). Cross-reactivity between ligands and receptors is a common feature for TGF-ß family members (Massagué, 1996a), in contrast to the high specificity of neurotrophins for their tyrosine kinase receptors (Piñón *et al.*, 1995).

The survival response of embryonic neurons from the PNS to neurotrophic factors is not static but changes throughout development. Although only neural crest-derived sensory neurons switch their response from BDNF and NT-3 to NGF during early stages of target field innervation (Buchman *et al.*, 1993; Buj-Bello *et al.*, 1994), changes in responsiveness to other trophic factors, like CNTF (Buj-Bello and Davies, unpublished data, Horton *et al.*, submitted) and GDNF has been shown to be a common feature of all PNS neurons. In chapter 2 of these thesis, it has been demonstrated that GDNF is able to support a wide variety of PNS neurons throughout development. Whereas sympathetic, parasympathetic and propioceptive neurons become less responsive to GDNF with age, enteroceptive and small- and large-cutaneous sensory neurons become more responsive to GDNF as development proceeds. Since neurturin exhibits a similar pattern of survival-promoting activity to GDNF on PNS neurons at a specific age, and E6 DMTG are unresponsive to both GDNF and neurturin, it will be important to analyse in detail the response of these neurons to neurturin during several stages of development and determine if the differences in potency between neurturin and GDNF are kept throughout development. The physiological relevance of changes in responsiveness to neurotrophins has been confirmed *in vivo* by studying the timing of neuronal death in the trigeminal ganglion of transgenic mouse embryos containing null mutations in the genes that encode the high affinity receptors for NGF, BDNF/NT-4/5 and NT-3 (Piñón *et al.*, 1996). Mouse trigeminal neurons are supported by BDNF and NT-3 at E11 and E12, and then switch their responsiveness to NGF

from E13 (Buchman and Davies, 1993). The peak of cell death in each tyrosine kinase receptor mutant coincides with the stage neurons are dependent on the specific ligand (Piñón *et al.*, 1996). Thus, it will be important to analyse *in vivo* the timing of neuronal death in GDNF and neurturin null mutant mice. However, prior to these experiments, it will be necessary to complete a comprehensive study on the effect of GDNF and neurturin on the survival of mouse PNS neurons during development, since only limited data are available about this subject at the moment. It was initially reported that GDNF promotes the survival of mouse E18 nodose ganglion neurons in culture (Henderson *et al.*, 1994). Preliminary data indicate that GDNF and neurturin both support the *in vitro* survival of mouse P4 nodose ganglion neurons (Piñón L.G., personal communication) and both are unable to promote the survival of P4 superior cervical ganglion neurons in culture (chapter 4 of this thesis). Furthermore, in GDNF null mutant mice there is a reduction in the number of neurons present in the nodose (40%), superior cervical (35%), and dorsal root ganglia (23%), indicating that GDNF is an important survival factor for these PNS neurons. It is to be expected that neurturin knockout mice will display a similar phenotype at the PNS level, although this remains to be elucidated.

In summary, it has been demonstrated that neurturin is a trophic factor with similar actions to GDNF in the developing avian peripheral nervous system. A more comprehensive survival study, including the analysis of the expression of neurturin mRNA in the target tissues of PNS neurons, will be necessary to determine the physiological relevance of this protein during neuronal development.

XI. CHAPTER 4

CLONING AND EXPRESSION OF CHICKEN GDNF AND NEURTURIN RECEPTORS ALPHA

1. INTRODUCTION

Glial cell line-derived neurotrophic factor (GDNF) is a trophic factor, initially purified and cloned from the rat glial cell line B49 (Lin et al, 1993), that exhibits a variety of biological activities. It has effects on several types of neurons from both the central and peripheral nervous system (for review, see Lapchak *et al.*, 1996), and on non-neuronal cells from tissues outside the nervous system.

Mice carrying targeted disruption of the GDNF gene have been recently generated. Homozygous mice display bilateral renal and ureteral agenesis, complete loss of the enteric nervous system, and a reduction in the number of neurons from peripheral ganglia and spinal motoneurons (Sanchez *et al.*, 1996; Pichel *et al.*, 1996; Moore *et al.*, 1996). The phenotype of GDNF null mutant mice is extremely similar to that observed in mice homozygous for a mutation in the RET gene, which display renal agenesis and hypodysplasia, and absence of enteric neurons throughout the digestive tract (Schuchardt *et al.*, 1994).

RET (rearranged during transfection) is a proto-oncogene that was first identified by transfection of NIH 3T3 cells with human T-cell lymphoma DNA; RET transforming potential was activated as a result of rearrangement between a DNA segment of RET and an unlinked DNA segment (Takahashi *et al.*, 1985). This DNA recombination gave rise to a chimeric oncogene that encoded a fusion protein with an amino-terminal region unrelated to any other known protein, and a carboxy-terminal region homologous to members of the tyrosine kinase gene family (Takahashi and Cooper, 1987). Full length human RET cDNA was subsequently cloned and sequenced, and encodes a receptor tyrosine kinase protein (RTK) (Takahashi *et al.*, 1988; 1989). The homologous mouse, chicken, and zebrafish RET proto-oncogenes have also been cloned (Iwamoto *et al.*, 1993; Schuchardt *et al.*, 1995; Marcos-Gutierrez *et al.*, 1997). Due to an alternative splicing in the 3' region of the gene, the Ret protein is expressed in two isoforms of different size, which differ only in their carboxy-terminal sequence (Tahira *et al.*, 1990). In

human, the long and short isoforms contain 1114 and 1072 amino acids, respectively. The first 1061 amino acids are identical between the two isoforms, but the 53 carboxy-terminal amino acids of the long isoform are absent from the short isoform and replaced by nine different ones (Takahashi *et al.*, 1989; Tahira *et al.*, 1990). The RET protein contains a large extracellular domain with no similarity to any other RTK. This domain includes a cadherin-like region distal to the membrane and a closer cysteine-rich region. The extracellular domain is followed by a single transmembrane domain, and an intracellular tyrosine kinase domain that is separated into two by a small insertion sequence.

Mutations in the human RET gene are involved in the pathogenesis of at least five diseases (for reviews, see Pasini *et al.*, 1996; Mak and Ponder, 1996). Three forms of somatic DNA rearrangement in RET have been described in papillary thyroid carcinomas (PTC) (Grieco *et al.*, 1990; Bongarzone *et al.*, 1993; 1994; Santoro, 1994). Furthermore, MEN2A (multiple endocrine neoplasia 2A), a rare inherited cancer syndrome that comprises medullary thyroid carcinoma, parathyroid hyperplasia and pheochromocytoma, and FMTC (familiar medullary thyroid carcinoma) are two diseases caused by germline missense mutations that affect conserved cysteine residues in the extracellular cysteine-rich domain (Mulligan *et al.*, 1993; 1994). These mutations result in constitutive activation of RET by ligand-independent dimerization of the protein (Santoro *et al.*, 1995). MEN2B, is an inherited and sporadic cancer syndrome that differs from MEN2A by the absence of parathyroid hyperplasia, and the presence of ganglioneuromas in the digestive tract, and skeletal and ophtalmic abnormalities. MEN2B is associated with a single germline missense mutation (met_{918} -> thr) in the catalytic core of the tyrosine kinase domain of RET (Hofstra t al., 1994; Carlson *et al.*, 1994), which results in a gain-of-function by a mechanism that remains unclear. Mutations in the RET gene have also been implicated in human Hirschsprung's disease (or aganglionic colon), an common inherited and sporadic disease characterized by the loss of parasympathetic neurons in the submucosal and myenteric plexi of the hindgut and the consequent lack of innervation of the colon (Romeo *et al.*, 1994; Edery *et al.*, 1994). Numerous mutations have been described and it is currently believed that, in some cases, the disease results from a loss of function of RET (Carlomagno *et al.*, 1996). However, RET mutations only account for 50% and 15-20% of familial and

sporadic Hirschsprung's disease cases, respectively. Other genes have been implicated in the ethiology of aganglionic megacolon such as the endothelin-B receptor (EDNRB) (Puffenberger *et al.*, 1994; Attié *et al.*, 1995), and its physiological ligand, endothelin 3 (EDN3) (Baynash *et al.*, 1994; Pasini *et al.*, 1996). GDNF is also mutated in some sporadic cases of aganglionic megacolon (Ivanchuk *et al.*, 1996), but not in inherited cases (Salomon *et al.*, 1996). Thus, Hirschsprung's disease may represent a multigenic disease.

Interestingly, RET null mutant mice and diseases that result from RET mutations are characterized by lesions that affect neural crest-derived tissues, indicating that RET plays an important role in the development and oncogenesis of neural crest derivatives.

Given the striking similarity between the phenotypes of RET and GDNF knockout mice, it was not surprising when GDNF was found to be the ligand of RET (Trupp *et al.*, 1996; Durbec *et al.*, 1996a; Vega *et al.*, 1996; Worby *et al.*, 1996). However, the fact that GDNF is a ligand for a receptor tyrosine kinase, and not a serine-threonine kinase like the rest of TGF-ß family members (Massagué, 1996b), was an unexpected finding.

It is currently believed that GDNF interacts indirectly with RET via a ligand-specific receptor named GDNF receptor alpha (GDNFR-α) (for reviews, see Mason, 1996; Robertson and Mason, 1997). GDNFR-α is a 468 amino acid protein that has been isolated by virtue of its GDNF binding in a screen of cDNA expression libraries derived from enriched populations of GDNF-responsive rat retinal photoreceptors (Jing *et al.*, 1996) and rat midbrain dopaminergic neurons (Treanor *et al.*, 1996). Human GDNFR-α has also been cloned, and shares 93% of identity to its rat homologue at the amino acid level (Jing *et al.*, 1996). GDNFR-α is a glycoprotein that contains 31 conserved cysteine residues and a stretch of 23 hydrophobic amino acids at the carboxy-terminus which mediates its binding to the cellular membrane via a glycosyl-phosphatidylinositol (GPI) linkage. GDNF binds to GDNFR-α with high affinity, with a dissociation constant (k_d) between 2.3×10^{-12} M (Jing *et al.*, 1996), and 63×10^{-12} M (Treanor *et al.*, 1996).

Since GDNFR-α does not contain either a transmembrane or intracellular domain, a second transmembrane protein must transduce the GDNF signal to the cytoplasm. It has been demonstrated that this protein is RET. GDNF induces RET

tyrosine phosphorylation in a dose-dependent manner, and RET mediates the biological activity of GDNF (Trupp *et al.*, 1996; Jing *et al.*, 1996; Vega *et al.*, 1996; this chapter). However, it remains unclear whether GDNF binds directly to RET. Although some studies have demonstrated a physical interaction between GDNF and RET, by co-immunoprecipitation of iodinated GDNF with anti-RET antibodies, and a low affinity binding of GDNF to RET (k_d of 0.2-9 x 10^{-9} M) (Trupp *et al.*, 1996; Vega *et al.*, 1996), other groups have been unable to obtain the same results and have shown that RET co-immunoprecipitates with GDNF only when GDNFR-α is present (Treanor *et al.*, 1996; Jing *et al.*, 1996). Furthermore, direct interaction between RET and GDNFR-α has been demonstrated by co-immunoprecipitation of GDNFR-α with RET antisera after GDNF treatment (Treanor *et al.*, 1996). Moreover, a RET extracellular domain/immunoglobulin Fc (RET/Fc) fusion protein can block RET activation, in a dose-dependent manner, when administered with a GDNF/soluble GDNFR-α preincubated mixture in a cell line that constitutively expresses RET (Jing *et al.*, 1996). These data strongly support a receptor complex model for GDNF signalling, that includes GDNFR-α and RET, probably as an heterotetradimer. According to this model GDNF binds specifically to GDNFR-α, and then this complex interacts with RET, which in turn dimerizes and is activated by tyrosine autophosphorylation.

Little is known about the intracellular signalling pathway triggered by activated RET. It has been shown that RET can bind and phosphorylate the SH2 domain of adaptor proteins such as Shc, She, Grb2, and Grb10 (Borello *et al.*, 1994, Pandey *et al.*, 1995; Arighi *et al.*, 1997; Lorenzo *et al.*, 1997). These molecules have been implicated in the Ras/MAPK pathway (for review, see Segal and Greenberg, 1996). Accordingly, GDNF has been shown to activate both MAPK and the transcription factor Elk (Worby *et al.*, 1996). Thus, it appears as if RET signals are transmitted to the cell nucleus by the MAPK pathway, however, other signalling pathways cannot be excluded.

RET is expressed in numerous embryonic tissues; it is present in the developing peripheral and central nervous systems, as well as in the renal system, including: cranial, dorsal root, sympathetic, and enteric ganglia, motoneurons, retina, the nephric Wolfian duct, and the ureteric bud epithelium. RET expression is also observed in the salivary gland, thymus, spleen, lymph nodes, and adrenal

chromaffin cells (Pachnis *et al.*, 1993; Tsuzuki *et al.*, 1995; Schuchardt *et al.*, 1994). A similar pattern of expression is found for GDNFR-α. GDNFR-α mRNA is detected in dorsal root ganglia, ventral midbrain and spinal cord, retina, developing nephrons and digestive tract, as well as in other regions of the CNS and non-neuronal tissues, including, thalamus, urogenital tract and pancreactic primordium (Treanor *et al.*, 1996).

Recently, a novel neurotrophic factor termed neurturin has been cloned (Kotzbauer *et al.*, 1996). It is structurally related to GDNF, and *in vitro* exerts similar survival-promoting effects to GDNF on PNS neurons (Kotzbauer *et al.*, 1996; chapter 3 of this thesis). Since neurturin and GDNF are homologous proteins that constitute a new family of neurotrophic factors, and GDNF signals through a ligand-specific receptor, it seems likely that the neurturin receptor is an homologous protein to GDNFR-α. By the end of writing up this thesis, the cloning of neurturin receptor alpha was reported (Klein *et al.*, 1997; Baloh *et al.*, 1997) and, in agreement with the data presented in this chapter, it is structurally related to GDNFR-α.

Thus, the aim of the study presented in this chapter was to clone the chicken homologue of GDNFR-α and putative related proteins, including the neurturin receptor. The screening of cDNA libraries with TrkA probes, using low stringency conditions, has proved to be an extremely useful tool for the cloning of its receptor homologues, TrkB (Klein *et al.*, 1989) and TrkC (Lamballe *et al.*, 1991). A similar approach was undertaken, and the cloning of both chicken GDNF and neurturin receptors, and their expression pattern are described.

2. CLONING OF CHICKEN GDNF AND NEURTURIN RECEPTORS ALPHA

2. 1. METHODS

2.1.1. Screening of a cDNA library

To isolate the chicken GDNF and NTN receptors alpha, a 1.5kb full coding region of the mouse GDNFR-α cDNA (gift of A. Rosenthal) was used as a probe to screen a once amplified E10 chicken brain cDNA library in Lambda Zap II vector (Stratagene) provided by V.L.Buchman. This library was chosen because when hybrising a Northern blot of total RNA from chicken tissues with the mouse GDNFR-α probe a single band of 10 kb was detected in several chicken embryonic tissues, including brain. This suggested that the putative chicken GDNFR-α mRNA or a related mRNA was expressed in embryonic brain (not shown). Before starting the screen, the library was titered by serial dilutions and was found to contain $6.5x10^7$ pfu/ml.

NOTE: all steps were carried out at room temperature unless otherwise stated. For the composition of the solutions used, see Sambrook *et al.*, 1989.

2.1.1.a) Plating and transferring the bacteriophage library

A culture of PKLF' Escherichia Coli bacteria was grown up to an OD_{600}=0.8 in YT broth containing 100 µg/ml tetracycline, 0.2% maltose and 10 mM $MgSO_4$. The bacterial culture was transferred to eight 6 ml tubes (0.6 ml of culture/tube) and mixed with 115 µl of 1:10 diluted recombinant phage stock solution, i.e. approximately 75,000 pfu/tube, and incubated for 20 minutes at 37°C for adsorption of phages to the bacteria. 6.5 ml of 0.7% YT top agarose contaning 0.2% maltose and 10 mM $MgSO_4$, preheated at 48°C, was added to each tube, and the mixture was immediately transferred onto 10x10 cm LB agar plates. The dishes were incubated at 37°C for a period of time between 8h to 10 h until clear lytic plaques appeared. Following this, the dishes were placed at 4°C for 1-2h before applying filters.

Phages were transferred to Hybon-N+ nitrocellulose filters (Amersham) (two replicas were made from each plaque) and denatured using the following solutions:

0.5N NaOH for 10 minutes, 1M Tris-HCl pH 7.5 for 1 minute, and 0.5M Tris-HCl pH 7.5 with 1.5M NaCl for 10 minutes. Phage DNA was then crosslinked to the membrane by a combination of UV irradiation (120,000 µjoules, Stratagene Stratalinker) and baking at 80°C for 2h.

2.1.2.b) Hybridization of recombinant phage DNA

The 1.5 kb insert of mouse GDNFR-α cDNA plasmid was radiolabelled by nick-translation (Sambrook *et al.*, 1989) and used as a probe for hybridization.

The filters were prehybridised for 1.5 hours at 59°C in plastic bags containing the following solution: 4xSSC, 0.2% SDS, 5mM EDTA, 15mM sodium phosphate pH 7.0, 5x Denhardt's solution and 100 µg/ml of salmon sperm DNA. Filters were transferred to new bags and hybridized for 20 hours at 59°C in the above solution containing the ^{32}P-labelled nick-translated mouse cDNA probe. Following hybridization, the solution containing the labelled probe was collected and kept at -20°C for use in further screenings. Then, the membranes were washed at low stringency conditions (three times for 15 minutes at 59°C in 2x SSC containing 0.2%SDS) before exposure to Kodak Omat X-ray film for 12 hours. Subsequently, filters were washed a second time at higher stringency. This entailed washing three times for 15 minutes at 65°C in 2x SSC with 0.2%SDS before exposure to Kodak Omat X-ray films for 20 hours.

Hybridization signals that were detected on duplicate autoradiographs from the higher stringency wash were marked. The corresponding areas, that contained the clones of interest, were collected from the agar dishes using a Pasteur pipette and transferred to Eppendorf tubes containing 0.5 ml of SM buffer (Ausubel *et al.*, 1995) plus 5 µl of chloroform. Phages were then stored at 4°C in SM buffer until use.

The phage suspension collected from this first screening was titered. In an attempt to isolate the desired clones, a second screening of the phages obtained from the first screening was performed, and finally, a third screening of the phages collected from the secondary screening was carried out. The plating, transfer, denaturation, and hybridization procedures were basically the same as described above, however a few changes were incorporated. The solution containing the ^{32}P-labelled nick-translated mouse cDNA probe that was made for the first screening

was used for both the second and third screenings. Following hybridization, filters were washed directly using high conditions of stringency, i.e. three times for 15 minutes at 65°C in 2x SSC with 0.2% SDS. The time of exposure of the films varied depending on the intensity of signals on the autoradiographs.

2.1.2. Isolation of recombinant cDNA

An *in vivo* excision method was used to isolate the recombinant cDNA (Sambrook *et al.*, 1989). The excision of the recombinant cDNA from the phage DNA and insertion into the Bluescript plasmid (pBS) was carried out by using the Exassist helper phage (Stratagene). For this, 200 µl of XL1-Blue MRF' E. coli bacteria at OD_{600}= 1 were mixed with 250 µl of phage stock solution from the tertiary screening, and with 1 µl of Exassist helper phage ($1x10^{10}$ pfu/ml), and incubated for 15 minutes at 37°C. This step allows the adsorption of phages to the bacteria. Then, 3 ml of LB broth were inoculated with this mixture and placed for 2-2h30, at 37°C, in a shaking incubator. During this time, the gene 2 protein from the helper phage is synthesized in the cell and nicks the (+) strand of the lambda bacteriophage DNA at the initiation site. As a result, the Bluescript plasmid and its insert DNA are replicated as a single-stranded DNA in a 5'->3' orientation. Some of these single-stranded molecules circularize and are packaged into phage particles, and secreted into the supernatant. After this period of incubation, 1 ml of the bacterial culture was transferred to fresh tubes and incubated for 15 minutes, at 70°C, to kill the bacteria. Following this, the tubes were centrifuged for 15 minutes at 4,000 rpm, to discard the bacteria. The supernatant, that contains the phage particles with single-stranded plasmid (or phagemid), was transferred to fresh tubes and stored at 4°C until use. Subsequently, either 100 µl or 10 µl of phagemid stock were mixed with 200 µl of XL1-Blue MRF' E. coli bacteria at OD_{600}=1 and incubated, for 15 minutes at 37°C, to allow adsorption of the phagemid to the bacteria. Finally, this mixture was plated on LB agar dishes that contained 50 µg/ml of ampicillin and incubated overnight at 37°C. During this time single-stranded plasmid DNA is converted into double-stranded DNA and replicates as a conventional plasmid. One single colony of bacteria was picked up from each plate and used to inoculated LB broth/ampicillin medium. Plasmid DNA was subsequently isolated from these bacterial cultures for analysis of the inserts.

2.1.3. Minipreps and midipreps of plasmid DNA

2.1.3.a) For restriction analysis

The plasmid used for restriction analysis was isolated from either small (2-5 ml) or medium (25-50 ml) volumes of bacterial cultures (minipreps or midipreps) by using a combined alkaline lysis/PEG procedure (Birnboim and Doly, 1979; Birnboim, 1983). Briefly, the bacterial pellet was resuspended and lysed in 100 µl of solution I*/ml of bacterial culture by vortexing. To denature bacterial proteins, chromosomal DNA and plasmid DNA, 200 µl of solution II*/ml of starting bacterial culture was added to the tubes and mixed. Following this, 150 µl of solution III*/ml of starting bacterial culture was added to neutralize the mixture. Most of bacterial proteins, chromosomal DNA and other cellular debris were eliminated by centrifugation and the plasmid DNA was precipitated by the addition of 1:1 vol. of isopropanol, at -20°C. The DNA pellet was washed in 70% ethanol, dried, and resuspended in 15 µl of distilled water/ml of starting bacterial culture. Two volumes of 7.5 M ammonium acetate was added to the DNA suspension, and the tubes were placed for 10 minutes at -20°C before being centrifuged again for 10 minutes at 15,300 rpm. The supernatant that contains the plasmid DNA was then precipitated by the addition of 1:1 vol. of isopropanol at -20°C. After centrifugation, the DNA pellet was washed in 70% ethanol, dried in a speed vacuum, and resuspended in 25 µl of distilled water/ml of starting bacterial culture. An equal volume of PEG-NaCl solution* was added to each tube, and after centrifugation for 10 minutes at 15,300 rpm, 1:1 vol. of PEG-NaCl solution was added again to the supernatant. The tubes were centrifuged for 10 minutes at 15,300 rpm, and the plasmid DNA pellet was washed in 70% ethanol and dried, before finally being resuspended in distilled water. The concentration of the plasmid DNA solution was measured by a combination of a spectrophotometer and ethidium bromide staining in agarose gels.

*Solution I: 50 mM glucose, 25 mM Tris.Cl pH 8.0, 10 mM EDTA and 10 mg/ml of hen egg white lysozyme. Solution II: 0.2 M NaOH and 1% SDS. Solution III: 5 M potassium acetate, pH 4.8. PEG-NaCl solution: 30% polyethylene (PEG 8000), 1.6 M NaCl.

2.1.3.b) For sequence analysis

For sequencing, miniprep DNA prepared by the boiling method was found to give good results and to be very quickly obtained (Holmes and Quigley, 1981). The procedure was as follows. Typically, 1.5 ml of overnight bacterial culture was transferred to Eppendorf tubes and centrifuged for 3 minutes at 6,000 rpm. Most of the supernatant was removed and cells were resuspended in the remaining 100 µl of supernatant by vortexing. 0.3 ml of STET solution* and 30 µl of hen egg white lysozyme (10 mg/ml in 25 mM Tris.Cl, pH 8.0) was added to each tube and mixed gently. The mixture was boiled for 90 seconds and transferred immediately to wet ice. The tubes were centrifuged for 10 minutes at 15,300 rpm, at 4°C, to discard cellular debris and chromosomal DNA, and the supernatant was transferred to fresh tubes. Then, the plasmid DNA was precipitated by adding 1:1 vol. of isopropanol and centrifugating for 10 minutes, at 15,300 rpm, at 4°C. The DNA pellet was washed with 70% ethanol and dried in a speed vacuum. The plasmid DNA was finally resuspended in 50 µl of distilled water and stored at -20°C until use.

* STET: 8% sucrose, 5% Triton X-100, 50 mM EDTA, 50 mM Tris-Cl pH 8.0.

2.1.4. Restriction analysis of cloned cDNA

The presence of an insert in the purified plasmids was assessed by restriction analysis. Digestion with EcoRI and XhoI released insert DNA from the plasmid vector. For a typical reaction, 1µg of purified plasmid DNA was incubated with both 1 µl of EcoR I (10u/µl, Promega) and 1 µl of Xho I (10u/µl, Promega) in buffer D (10x buffer: 60 mM Tris-Cl pH 7.9, 60 mM $MgCl_2$, 1.5 M NaCl, 10 mM DDT, Promega) in a final volume of 20 µl. The mixture was incubated for 2 hours at 37°C. Subsequently, the samples were run in a 1% agarose electrophoresis gel using TAE buffer (for composition see, Sambrook *et al.*, 1989).

2.1.5. Southern blotting and hybridization of cloned cDNA

After gel electrophoresis, the DNA fragments resulting from the restriction reaction were blotted onto an Hybond-N+ nitrocellulose membrane (Amersham). Prior to blotting, the DNA from the gel was denatured by washing the gel in 0.5 M NaOH with 1.5 M NaCl for 40 minutes, followed by washing in 0.5 M Tris-HCl pH 7.2 with 3 M NaCl for another 40 minutes. Subsequently, the denatured DNA

was transferred onto Hybond-N+ filters, and crosslinked by both UV irradiation (120,000 µl, Stratagene Stratalinker) and baking at 80°C.

The filters were prehybridized, as described in the section 2.1.1.b), for 30 minutes at 60°C, and then hybridized for 12 hours at 60°C in the same solution containing ^{32}P-labelled mouse GDNFR-α cDNA that was previously used for hybridizing filters from the library screening. After hybridization, the filters were washed three times for 15 minutes, at 65°C, in 2x SSC with 0.2% SDS, before exposure to Kodak films overnight.

2.1.6. DNA sequencing

DNA sequencing of the cloned inserts was performed by the termination-chain reaction, or dideoxy method (Sanger *et al.*, 1977), using the version 2.0 DNA Sequencing Kit (USB).

2.1.6.a) Alkali denaturation of double-stranded DNA

8 µl of plasmid DNA (2 µg), purified by the boiling method, was denatured by adding 2 µl of 2 M NaOH with 20 mM EDTA in a final volume of 20 µl and incubated for 20-30 minutes at 37°C. The mixture was then neutralized by adding 6 µl of 7.5 M ammonium acetate. The denatured DNA was precipitated by adding 100 µl of ethanol and placing the tube at -20°C for at least 1hour. The tubes were then centrifuged for 10 minutes at 15,300 rpm, at 4°C, and the DNA pellet was washed with 70°C ethanol and dried under vacuum.

2.1.6.b) Annealing reaction

The denatured plasmid DNA pellet was resuspended by vortexing in 7 µl of distilled water, 1µl of synthesized primer (100 ng/ µl), and 2 µl of 5x sequenase reaction buffer (USB kit). The mixture was then incubated for 15-20 minutes at 37°C, and immediately transferred to wet ice.

2.1.6.c) Labelling reaction

To each tube containing the annealed template-primer the following solutions were added: 1 µl of 0.1 M DTT, 2 µl of 1:5 Labelling Mix (USB kit), 0.5 µl of ^{35}S-dATP (12.5 mCi/ml), 2 µl of 1:8 Sequenase DNA Polymerase version 2.0 (USB

kit). During this procedure, the tube was always kept in wet ice. The mixture were then mixed gently and incubated at room temperature for 2 minutes. Finally, the tubes were centrifuged for 15 seconds to spin down the solution.

2.1.6.d) Termination reaction

For each sequencing reaction, 4 tubes were prepared by adding 2.5 µl of either ddATP, ddTTP, ddCTP or ddGTP Termination Mix (USB kit). These tubes were pre-warmed at 37°C. Subsequently, 3.5 µl of labelling reaction was transferred to each termination tube and mixed. The mixture was incubated for 5 minutes at 37°C. Then, the reaction was stopped by addition of 4 µl of Stop Solution (USB) to each termination tube followed by mixing.

Prior to electrophoresis, the DNA for the sequencing reaction was denatured by heating at 85°C for 15 minutes and immediately transferring to wet ice.

2.1.6.e) Gel electrophoresis and autoradiography

Electrophoresis of radiolabelled sequencing products was carried out in a 17x50 cm 6% denaturing polyacrylamide gel using TBE buffer (for procedure see, Sambrook *et al.*, 1989). Samples were usually run for 3-4 hours, at 50 W constant power, although shorter runs were also used when required. After electrophoresis, the gel was fixed by a combination of treatment in 10% acetic acid for 1 hour and baking for another hour at 80°C. Kodak BioMax X-ray films were exposed to the sequencing gels for aproximately 12 hours.

Note: the primers used for sequencing were made using an Applied Biosystems 391 DNA Synthesizer according to manufacturer's instructions. The oligonucleotides were usually 15 bp long, with a composition 1:1 of C/G:A/T.

2.2. RESULTS

2.2.1.Isolation and sequence of chicken GDNFR-α and NTNR-α cDNAs

An E10 amplified chicken brain cDNA library was screened at low stringency using the full coding region of mouse GDNFR-α as a probe. From 600,000 recombinant phages analysed, thirty one plaques which hybridized with the probe were detected in the first screening (1 positive/19,000 pfu). Twelve of these clones were further analysed. *In vitro* excision was carried out and cDNAs cloned into the plasmid Bluescript were isolated. Two sets of overlapping cDNAs were identified using restriction endonuclease mapping of these plasmids. The first set of clones contained eight overlapping cDNAs of 2.3 to 3.5 kb in which two internal EcoRI were present. The second set of clones contained four overlapping cDNAs of 2.5 to 2.9 kb in which no internal EcoRI site was present. The nucleotide sequence of these clones was determined by the termination-chain reaction.

The first set of overlapping cDNA clones corresponded to GDNFR-α cDNA (as explained in next section). The two DNA strands were sequenced and the nucleotide sequence of the 5'->3' strand is shown in figure 4.1. The length of this cDNA is 3037 bp. A C/G rich 5' region of 739 bp is followed by a single open reading frame (ORF) of 1410 bp that extends from nucleotide 740 to the termination codon TAG at nucleotide 2149. This termination codon is followed by an A/T rich 3' untranslated region (from nucleotide 2150 to 3037) which contains a putative consensus polyadenylation signal (AATAAA) 33 nucleotides upstream of the poly-A tail.

The second set of overlapping cDNA clones corresponded to NTNR-α cDNA (as explained in next section). The cloned cDNA contains 2933 bp. The two DNA strands were sequenced by the termination-chain reaction, and the sequence of the 5'->3' strand is shown in figure 4.2. In this cDNA, there is a very short 5' region followed by a single ORF of 1398 bp that extends from nucleotide 38 to the stop codon TAG at nucleotide 1435. The termination codon is followed by a long A/T rich 3' region of 1501 bp. The sequence contains a putative consensus polyadenylation signal (AATAAA) 26 nucleotides upstream of the poly-A tail.

Figure 4.1. Nucleotide sequence of the chicken GDNFR-α cDNA corresponding to the 5'->3' strand. The open reading frame is indicated in bold. Nucleotides are numbered in the right-hand margin of the figure.

```
cgagcgcgccccctgaggcagcggggctcctcctgaggcgcgggcagcgc  50
accgccgtctcagtcaccgggctgcggctacccgtcgggagccccccctgc  100
tgcgcccgacctgccccgccggagcagctccctgcaggtgacggagctcc  150
ctcccagtttgcccggctaaactttcccagctgccggcagcacctgtgcg  200
tatctcccgggcgagcggcacgaccgcctccgcgcgaagaaataaagttg  250
cggctttgaggaggggacgaatcccttcctgcagggctcgggcactcgcg  300
gggagccccgtaggtgcgtgcggggcgtcagcgctcttcccccacccct  350
ccccgtgagcgctcggtccctccccgggcacaggctgctgccacccaagg  400
aggcgcccggctgctgccccgctgccgccgctccgtgcacacacacagac  450
acacaaatacggtgcgttgcgcctccgggagtcagcgcctccgccttcag  500
gttgctcagacctgaaatctacggggaatctcagcgttcttgtcgccgct  550
cctgccggtggaagcggtgaagaggagagattttgatcattatcattatt  600
attgttatttccccttcttatatcaatggatcggaacttggagtctttg  650
cacctcggcggttttggaatatctacatgctgagcctctttgttgtggca  700
catcggctcagttcgggggaaccatccggagacggcacg atgttcctcgc  750
gctcctctacttggctctgcccttagcggacgtacttctgtcggcagaag  800
tcagcgggctgcccggaggggaccgcctcgactgcgtgaaagccagcgat  850
cagtgtctcaaggagcagagctgtagtactaagtacaggacactgaggca  900
gtgtgtagccggcaaagagagcaacttcagccgggcgacgggcctggagg  950
cgaaggatgaatgcaaaagcgccatggaggctctcaagcagaaatctctg  1000
tacaactgccgctgcaagaggggcatgaagaaggagaaaaactgcctgcg  1050
catttactggagcatgtaccagagcttacagggaaatgacttgcttgagg  1100
attctccctatgaaccagttaacagcaggctatcagacatattcaggcta  1150
gcaccgattgtatcagtggagccagtactatcaaaggggaacaactgctt  1200
ggatgcagcaaaagcttgtaacctaaatgatacctgcaagaggttcagat  1250
ctgcttacataaccccctgcaccagcagcacgtctaatgaaatctgtaac  1300
aagcggaagtgtcataaggccctccggctatttttgacaaagttccccc  1350
aaagcacagctacgggatgctcttctgctcctgtcgagacgtagcctgta  1400
cagaaaggaggcggcagactattgttcctgtgtgttcatatgaggacagg  1450
gagaaaccaaactgcctgaatttacaagaatcctgcaagaagaattacat  1500
ctgcagatctcgccttgcagatttttcacaaactgccagcctgagtcac  1550
gctctgttagtagctgtctgaaggagaactacgctgactgcctcctcgct  1600
tactcggggctcattggcacagtgatgacaccgaactacatagactcaag  1650
tagtctcagcgttgccccatggtgtgactgcagcaacagtggtaatgaca  1700
tagatgaatgccggaaatttctgaatttcttccaggacaacacatgcctt  1750
aaaaatgcaattcaggctttcggcaatggtactgatgtgaatgtgtggca  1800
gccaatattaccggtacagaccactacagccacaactacaacagcttcca  1850
gacttaaaaacacaggttcagagaccaccaacaatgaaatacccacccac  1900
aatgattcaccagcatgtgcgaacctgcaggcacagaagaagcggaaatc  1950
caatgaatctgtagatacagaactctgtcttaatgagaatgctattggga  2000
aagacaacacaccaggagtctccaccagccacatatcctcggagaattct  2050
tttgcccttcctacaagcttctatccaagcacaccactcattctgatgac  2100
aattgcactctcactcttttgttcctaagctcatcagtcgtcttgtagc  2150
tgcattgcaaaaggacatgtaaaaaaatctgtttcctgtcctctgttgtt  2200
tatctggaattccaggtctgggggctaagctgaggcacttctgctagaac  2250
agtttgtcagctggaaaaatttttctctctaaaaaagcttcttgtgata  2300
tttagaggctttgtgaatacttggtgcagtgctacattccaaacccaaga  2350
ggcttttgggcatgcagtgttttgaagagacagtgtataaatttgcctgt  2400
aaagagatctggttggattattttaataattatattaattctggccttta  2450
cctgagaaggaggatggcagttttcttaagatcctatttatctcattgga  2500
tggttttggttttcaaattgatcaaactccagattatcaaggatgtcagg  2550
cttttgtctaatggtgaatgttctcccagagagtggactttatgaaactt  2600
```

cttcatttgataaattgctactgatgttaaactctttcagtgtattagca 2650
ttttcctctttaaatgtttacgtactgtaagtgattctgcgttccctgct 2700
gagaagccattctaattcacatacaggtgtaacgtatgtctttcagttaa 2750
attcttatagagtgtggcatagaactttctaacaaaacatttatctttta 2800
attataatcatctagccttaacgagggtgaagattctttaaattaacaag 2850
aagcagccattgtgaaagctccgtaaacgtacatttcataaactttgagg 2900
atgaacagtagaaaaaaattttgctgcagggtttcagctgtacagtcacc 2950
ggtcttctgtgcttccattgtgacaatatagtccaattattatactgttc 3000
attcaataaaaaagatatgcattttacttgctctact 3037

Figure 4.2. Nucleotide sequence of the chicken NTNR-α cDNA corresponding to the 5'->3' strand. The open reading frame is indicated in bold. Nucleotides are numbered in the right-hand margin of the figure.

```
cctaaacgcacgtggaggaactcgtggatgtactaacatgattttggcca  50
acgccttctgcatcgtcctctttgtagatgagaccctccgctcgctggcc  100
gccccccgtccccccccgggcaggacctgcagggctggcgggtgccggt  150
ggactgcatacgtgccaacaagctgtgtgcagccgagggcagctgcagct  200
cccggtaccgcaccctgcggcagtgcctggcgggacgcgaccgcaacacc  250
atgctggccaacaaggagtgccaggcggccctggaggtgctgcaggagag  300
cccgctgtacgactgccgttgcaagaggggcatgagaaaggagattcagt  350
gccttcaggtctactggagtatacacctcgggctggccgaaggagaagag  400
ttttacgaagcttcccctacgagccgatcacctctcgtctctctgatat  450
attcagactcgcttcaattttctcaggaatggaccctgccaccaattcca  500
aaagcaaccactgcctcgacgcggccaaagcgtgcaacctgaacgacaac  550
tgcaagcgcctgcgctcgggctacatctccacctgcagcaaggagatctc  600
ggccaccgagcactgcagcaggaggaaatgccacaaagccctgcgccagt  650
tcttcgacaacgtgcccagcgagtacacctaccgcctcctcttctgctcc  700
tgcaaggaccaggcgtgcgccgagccgcggcggcaaaccatcgtcccctt  750
ctgctcctacgaggacaaggagaaacccaactgcttggatctgcgcaacg  800
tgtgccgcgccgaccacctgtgccggtcccggctggctgatttccacgcc  850
aattgccaggcctccttccagtcactgaccagctgccctggggacaacta  900
tcaggcgtgcctgggctcctacacagggctcattggttttgatatgacgc  950
ccaactacgtggatgccagcaccaccagcatcactatctcgccctggtgc  1000
tcctgcaaaggcagcggcaacttggaggaggagtgtgagaagttcctgcg  1050
ggacttcactgagaacccctgtctccgaaatgctatccaagccttcggca  1100
atggcaccgatgtcaacctctcccccaagaacccctcacctcccatcaca  1150
atgctgcccaaggtggagaaaagtcctgccttgcctgatgacatcaacga  1200
cagcaacaccatgtacgacacgagcatcatcaccacctgcacctccatcc  1250
aggagcacggacagaagctaaacaagtccaaagagcagagcctgtgctac  1300
tcagagacccagctcaccacggacacgatgccagaccagaagacctttgt  1350
ggatcagaaggcagccggcagccggcaccgggcagcccggatccttccag  1400
ctgtgcccattgtgctgctgaagctgctgctataggggacaattgccgca  1450
tctcagctccggagtgctgggctcatctcgggctccagactgcaagcgga  1500
acaccagcgatgcccaaggccagaaggaccctcacagtgatgggaaactt  1550
cagtggttttcttttttttttttccttttccttctttttttttggcctt  1600
tttgttcgtttgatggtgcgttgggtttctactgctggagcacgagcccg  1650
gcacaacccacccaggcaaccacaaaggggaaccaatgcaatcaacggca  1700
ccggggagggagaaaggcagcgagcagagctgtgggatgagggctgtct  1750
gcagggctaccaacccagcgtgctgagggacccgttggggacgtgggacg  1800
tttgcgcatcgccgcggcacagcggtgccattggggctcctttgggcaca  1850
gactctccgctgccccacgtcgtccccgggactgttttcttttccttcc  1900
cgtggttggtgagagttttcaaatcagaaaagaaaccaatattcaaacgg  1950
tgtgaggctgtcctgatgttttctatctgtcactcggaggggaggtttt  2000
ccttttattatttaccctttctttttcattatgcctttctttcccatct  2050
tcattccatcggatttgggttgggatgggatccgaccccagcggcggttg  2100
gagtcctctgtatgttactcagttttcttttatatatacatatatagtgt  2150
atgtatatagcgctcacctatacctacagctatgtatgtatagacgtaac  2200
catagagatctaccggcgtggctctctaaggtttcctttcaaaaaaggag  2250
ggctgcaaaatgtatgattgtaaagtttatttttttaaacgatgtctat  2300
aatggaaataataataataattaataataataataataaaaggagaa  2350
gaaagaaccaagaggagaacaaagcaccacctgaggggaggaaggggggg  2400
gtccgacgtcccatagagcatcccaagccctggctgcatgctgggggatg  2450
ctcagctatagagaggcacagatttgggtcaaccccacacgtcttgcagt  2500
gctgcaggacgcctcgcctggctgtttggctctgggagacgtgcaaacaa  2550
gcaaacatttcaaaggagctgacaggttgctcgcgtggcagagctctttt  2600
```

```
ttctttaaagcacctcatcctccgtgctgcttctttttccccacccccca  2650
gtgcttaaaacccaaacgaaggttgaaatttgggtcacgcaaaacctcgt  2700
gttcctcctcctcccccctttccccagtcagaagtccttttcccaccttct  2750
gctcgcaccgtgcagcagcagcagcaccgacccgcaggcagctctgcgca  2800
gtggggctccaaggctgccgagaccccattgcaaaaggaggggcaaatct  2850
tccccttgcaaagcagggagcgaaaacacgtttgatgtgcaagttggca  2900
cggggaaaataaacatgtcacgtctctcgccgg  2933
```

2.2.2. Protein sequence of chicken GDNFR-α and NTNR-α

Chicken GDNFR-α cDNA encodes a predicted protein of 459 amino acids (Software, DNA Strider). Sequence comparison with the published rat GDNFR-α (Treanor *et al.*, 1996; Jing *et al.*, 1996) indicates that the chicken protein has 80 % identity with its rodent homologue, including conservation of all 30 cysteines (figure 4.3). This indicates that they are structurally homologous proteins. Whereas the carboxy-terminal region of chicken GDNFR-α shares only 45% identity with rat, the rest of the protein is highly conserved between birds and mammals (87% identity within the first 375 amino acids).

Another cDNA clone, different from GDNFR-α, was obtained from screening the same library with the same probe as described above. This cDNA encodes a protein of 465 amino acids, named for reasons given below NTNR-α. This protein has 49% identity with chicken GDNFR-α, including all 30 cysteines localised at the same relative positions (figure 4.4). Search in the database have shown that this is a novel protein.

Chicken GDNFR-α and NTNR-α both possess an hydrophobic amino-terminal domain of 13 amino acids, that corresponds to a putative signal peptide for secretion, followed by a cysteine rich domain (figure 4.4). The carboxy-terminal region of both GDNFR-α and NTNR-α shows the characteristic features of glycosyl-phosphadidylinositol (GPI)-linked proteins: a carboxy-terminal hydrophobic domain (23 and 14 amino acids, for GDNFR-α and NTNR-α, respectively) separated by an hydrophilic linker region from a cleavage/binding consensus sequence for GPI linkage (Ser_{428} Thr_{429} Ser_{430} for GDNFR-α, and Ala_{443} Gly_{444} Ser_{445} for NTNR-α) (Gerber *et al.*, 1992). These features suggest that both GDNFR-α and NTNR-α are secreted proteins attached to the extracellular surface of the plasma membrane by a GPI-linkage. Moreover, these data indicate that GDNFR-α and NTNR-α form a novel family of homologous proteins.

Figure 4.3. Aligned protein sequences of rat and chicken GDNFR-α.
Identical amino acids are marked with asterisks and the conserved cysteines are enclosed in vertical boxes. The amino-terminal, hydrophobic, putative signal peptides are underlined. Rat GDNFR-α sequence was obtained from Treanor *et al.*, 1996.

```
rat GDNFR-α  MFLATLYFALPLLDLLMSAEVS---GGDRLDCVKASDQCLKEQSCSTKYR  47
             **** ** **** * * *****    ********************** 
ch. GDNFR-α  MFLALLYLALPLADVLLSAEVSGLPGGDRLDCVKASDQCLKEQSCSTKYR  50

rat GDNFR-α  TLRQCVAGKETNFSLTSGLEAKDECRSAMEALKQKSLYNCRCKRGMKKEK  97
             **********  ***    ********* ********************** 
ch. GDNFR-α  TLRQCVAGKESNFSRATGLEAKDECKSAMEALKQKSLYNCRCKRGMKKEK  100

rat GDNFR-α  NCLRIYWSMYQSLQGNDLLEDSPYEPVNSRLSDIFRAVPFISDVFQQVEH  147
             ********************************  *  *     ** 
ch. GDNFR-α  NCLRIYWSMYQSLQGNDLLEDSPYEPVNSRLSDIFRLAPIVS-----VEP  145

rat GDNFR-α  -ISKGNNCLDAAKACNLDDTCKKYRSAYITPCTTSMSNEVCNRRKCHKAL  196
              **************  ****  *********  * *** **  ********* 
ch. GDNFR-α  VLSKGNNCLDAAKACNLNDTCKRFRSAYITPCTSSTSNEICNKRKCHKAL  195

rat GDNFR-α  RQFFDKVPAKHSYGMLFCSCRDIACTERRRQTIVPVCSYEERERPNCLSL  246
             * ****** ********** ********************** ** **** * 
ch. GDNFR-α  RLFFDKVPPKHSYGMLFCSCRDVACTERRRQTIVPVCSYEDREKPNCLNL  245

rat GDNFR-α  QDSCKTNYICRSRLADFFTNCQPESRSVSNCLKENYADCLLAYSGLIGTV  296
             * *** ***********************  ********************** 
ch. GDNFR-α  QESCKKNYICRSRLADFFTNCQPESRSVSSCLKENYADCLLAYSGLIGTV  295

rat GDNFR-α  MTPNYVDSSSLSVAPWCDCSNSGNDLEDCLKFLNFFKDNTCLKNAIQAFG  346
             *****  ********** ********    * ****** ********** 
ch. GDNFR-α  MTPNYIDSSSLSVAPWCDCSNSGNDIDECRKFLNFFQDNTCLKNAIQAFG  345

rat GDNFR-α  NGSDVTMWQPAPPVQTTTATTTTAFRVKNKPLGPAGSENEIPTHVLPP-C  395
             ** **  ***  ************ * **          ******   * * 
ch. GDNFR-α  NGTDVNVWQPILPVQTTTATTTTASRLKNTGSETTN--NEIPTHNDSPAC  393

rat GDNFR-α  ANLQAQK-LKSNVSGSTHLCLSDSDFGKDGLAGAS-SHITTK-SMAAPPS  442
             *******  *** *  * ***     ***   * * ***    * * * * 
ch. GDNFR-α  ANLQAQKKRKSNESVDTEICLNENAIGKDNTPGVSTSHISSENSFALPTS  443

rat GDNFR-α  CSLSSLPVLMLTALAALLSVSLAETS                          458
                *    **  **   *  *
ch. GDNFR-α  FYPSTPLILMTIALSLFLFLSSSVVL                          459
```

Figure 4.4. Aligned protein sequences of chicken GDNFR-α and NTNR-α. Identical amino acids are marked with an asterisks, and the conserved cysteines are enclosed in vertical boxes. The amino-terminal, hydrophobic, putative signal peptides are underlined. The carboxy-terminal hydrophobic domain is double-underlined and the putative binding/ cleavage consensus sequences for GPI linkage are enclosed in horizontal boxes. Gene Bank accession numbers: chicken GDNFR-α, U90541; NTNR-α, U90542.

```
GDNFR-α   MFLALLYLALPLADVLL-S--AEVSGLPGGDRL-------DCVKASDQCL  40
          * **          *  * * *  *  ** * *        **  *   *
NTNR-α    MILANAFCIVLFVDETLRSLAAPPS-PPGQD-LQGWRVPVDCIRANKLCA  48

GDNFR-α   KEQSCSTKYRTLRQCVAGKESNFSRATGLEA-KDECKSAMEALKQKS-LY  88
           * ***  ******* **   *    * * * * **  * * * * * **
NTNR-α    AEGSCSSRYRTLRQCLAGRDRN----TML-ANK-ECQAALEVL-QESPLY  91

GDNFR-α   NCRCKRGMKKEKNCLRIYWSMYQSL-QGNDLLEDSPYEPVNSRLSDIFRL 137
          ********* **  **  ***   *  *   * *****  *********
NTNR-α    DCRCKRGMRKEIQCLQVYWSIHLGLAEGEEFYEASPYEPITSRLSDIFRL 141

GDNFR-α   APIVSV-EPVL-SKGNNCLDAAKACNLNDICKRFRSAYITPCTSSTS-NE 184
          * * *   *   ** * ********** **** ** **  *    * *
NTNR-α    ASIFSGMDPATNSKSNHCLDAAKACNLNDNCKRLRSGYISTCSKEISATE 191

GDNFR-α   ICNKRKCHKALRLFFDKVPPKHSYGMLFCSCRDVACTERRRQTIVPVCSY 234
           *  ********  *** **    *  ****** * **  * ******* ***
NTNR-α    HCSRRKCHKALRQFFDNVPSEYTYRLLFCSCKDQACAEPRRQTIVPFCSY 241

GDNFR-α   EDREKPNCLNLQESCKKNYICRSRLADFFTNCQPESRSVSSCLKENYADC 284
          ********* *   *       ********  ***    *  **   **  *
NTNR-α    EDKEKPNCLDLRNVCRADHLCRSRLADFHANCQASFQSLTSCPGDNYQAC 291

GDNFR-α   LLAYSGLIGTVMTPNYIDSSSLSV--APWCDCSNSGNDIDECRKFLNFFQ 332
          *  * ****  ***** * *  *    * * *  ***   **  ***  *
NTNR-α    LGSYTGLIGFDMTPNYVDASTTSITISPWCSCKGSGNLEEECEKFLRDFT 341

GDNFR-α   DNTCLKNAIQAFGNGTDVNVW--QPILPVQTTTATTTTASRLKNTGSETT 380
           * ** ************     *  *  *         *
NTNR-α    ENPCLRNAIQAFGNGTDVNLSPKNPSPPI-TMLPKVEK-SPALPDDINDS 389

GDNFR-α   NNEIPTHNDSPACANLQAQKKRKSNESVDTELCLNENAIGKDNTPGVSTS 430
          *    *       *   *     * * *    ** *     *  *   *
NTNR-α    NTMYDTSI-ITTCTSIQ-EHGQKLNKSKEQSLCYSETQLTTDTMPDQKTF 437

GDNFR-α   HISSENSFALPTS-FYPSTPLILMTIALSLFLFLSSSVVL           469
                         *  *  *    *
NTNR-α    VDQKAAGSRHRAARILPAVPIVLLKLLL                       465
```

3. FUNCTIONAL ANALYSIS OF CHICKEN GDNF AND NEURTURIN RECEPTORS ALPHA

Comparative analysis of chicken GDNFR-α and rat GDNFR-α protein sequences has shown that they are homologous proteins with 80% identity (this chapter). Rat GDNFR-α is a GPI-linked ligand-specific receptor that binds with high affinity to GDNF and associates with the transmembrane protein tyrosine kinase RET to form a receptor complex that transduces GDNF signal to the cytoplasm of the cell (Trupp *et al.*, 1996; Durbec *et al.*, 1996a; Jing *et al.*, 1996; Treanor *et al.*, 1996; Vega *et al.*, 1996; Worby *et al.*, 1996). Thus, it is likely that cloned chicken GDNFR-α binds to the same ligand as its rodent homologue.

A novel neurotrophic factor named neurturin (NTN) has been recently purified and cloned. Neurturin contains 42% identical amino acids to GDNF (Kotzbauer *et al.*, 1996). The aim of this study is to ascertain whether GDNF and neurturin are the ligands of cloned chicken GDNFR-α and the GDNFR-α homologue termed, for reasons given below, NTNR-α.

3.1. EFFECT OF PHOSPHOLIPASE C ON CULTURED PNS NEURONS

Proteins bound to the membrane by a glycosyl-phophatidylinositol (GPI) anchor can be readily distinguished from other membrane-associated proteins by the use of the enzyme phosphatidylinositol-specific phospholipase C (PIPLC) which specifically cuts these proteins free from their anchors and thereby releases them from the membrane. This approach was used to determine whether GDNF and neurturin are both neurotrophic factors that mediate their biological action via a GPI-linked receptor. Part of the data presented in this section was incorporated into the publication by Treanor *et al.*, 1996.

3.1.1. Methods

E6 nodose and E14 sympathetic lumbar chain ganglia were dissected from chicken white leghorn embryos. The neurons of the latter ganglia were separated from non-neuronal cells by differential sedimentation (Davies, 1988a). Cells were

grown in the 10 mm diameter wells of 4-well 35 mm plastic tissue culture dishes (Nunc, Gibco) (as described in chapter 2).

4 μg/ml of PIPLC (a gift from A. Rosenthal) was added to the culture medium of some of the dishes 1.5 hours before and 12 and 24 hours after addition of neurotrophic factors (either BDNF, NGF, GDNF or NTN). The number of surviving neurons was determined 36 hours after plating and expressed as a percentage of the number plated (as described in chapter 2).

3.1.2. Results

BDNF, GDNF and neurturin responsive E6 chicken nodose ganglion neurons were cultured in the presence or absence of PIPLC (4 μg/ml) for 36 hours. The number of neurons surviving with GDNF at either 50 ng/ml or 1 μg/ml was clearly reduced when neurons were grown in the presence of PIPLC (figure 4.5). Similarly, PIPLC treatment reduced the number of neurons surviving with either low concentrations of neurturin (5 ng/ml), from over 70% to less than 10% (figure 4.6), or high concentrations of neurturin (1 μg/ml), from about 70% to 45 %. This reduction in neuronal survival was not due to a neurotoxic effect of PIPLC, because the survival response of nodose neurons to BDNF, which signals via a transmembrane TrkB receptor tyrosine kinase (Klein *et al.*, 1989), was unaffected by PIPLC (figures 4.5 and 4.6).

To ascertain whether PIPLC exerts a similar action on other types of neurons than the BDNF responsive nodose ganglion neurons, E14 sympathetic lumbar chain neurons were grown in the presence of PIPLC. The number of surviving neurons with high concentrations of GDNF (1 μg/ml) was greatly reduced, from about 70% to 25%, in the presence of PIPLC (figure 4.7). However, PIPLC treatment did not affect the number of neurons that survived with NGF, which, like BDNF, signals via a transmembrane receptor, the TrkA receptor (Martin-Zanca *et al.*, 1989).

These results suggest that the survival-promoting effects of GDNF and neurturin on PNS neurons is mediated by GPI-linked receptors.

Figure 4.5. Effect of PIPLC treatment on E6 chicken nodose ganglion neurons cultured in the presence of either GDNF or BDNF. Bar chart of the percent survival of E6 chicken nodose ganglion neurons grown with either BDNF (10 ng/ml) or GDNF (GDNF 1: 50 ng/ml or GDNF 2: 1 μg/ml) for 36 hours in the presence or absence of PIPLC. The means and standard errors are shown (n=4).

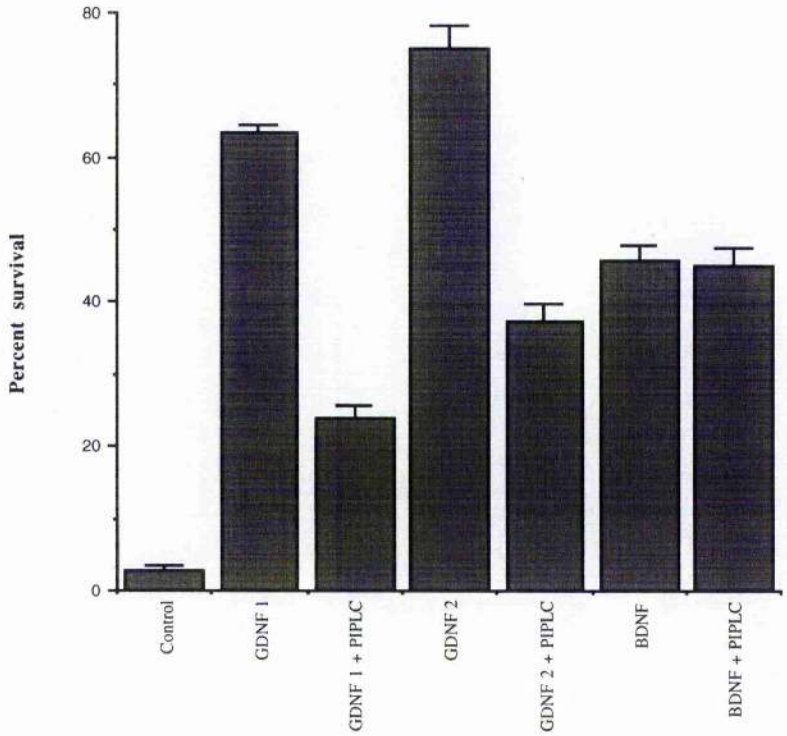


Figure 4.6. Effect of PIPLC treatment on E6 chicken nodose ganglion neurons cultured in the presence of either neurturin or BDNF. Bar chart of the percent survival of E6 chicken nodose ganglion neurons grown with either neurturin (5ng/ml) or BDNF (5ng/ml)) for 36 hours. Half of the cultures were treated with PIPLC. The means and standard errors are shown (n=4).

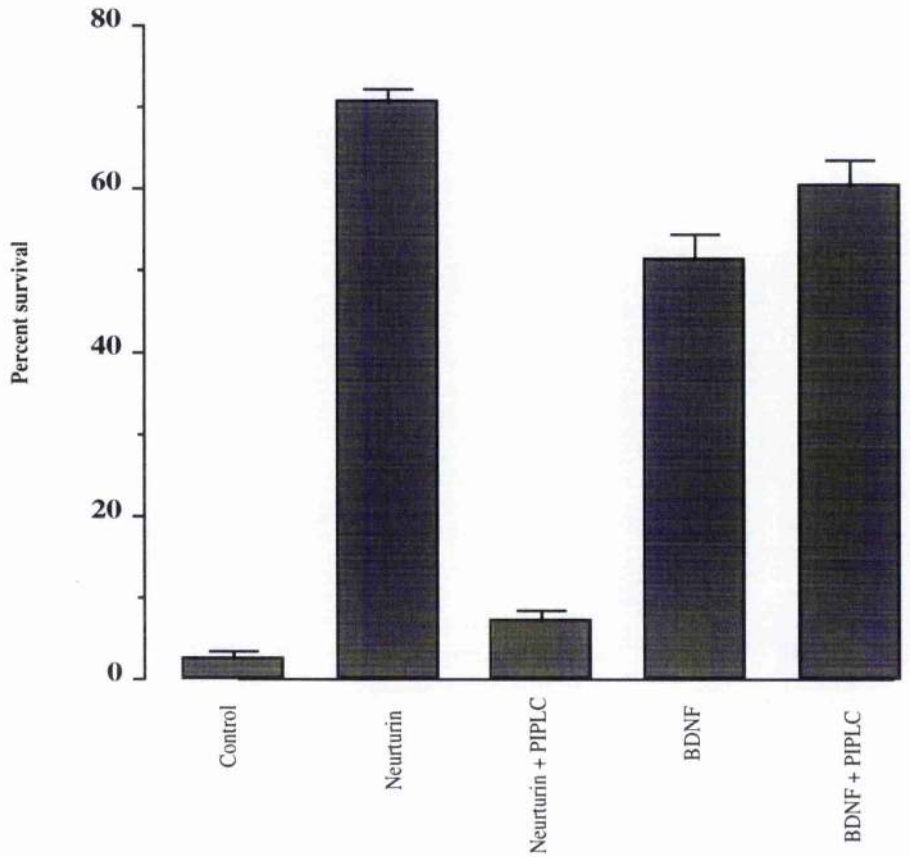
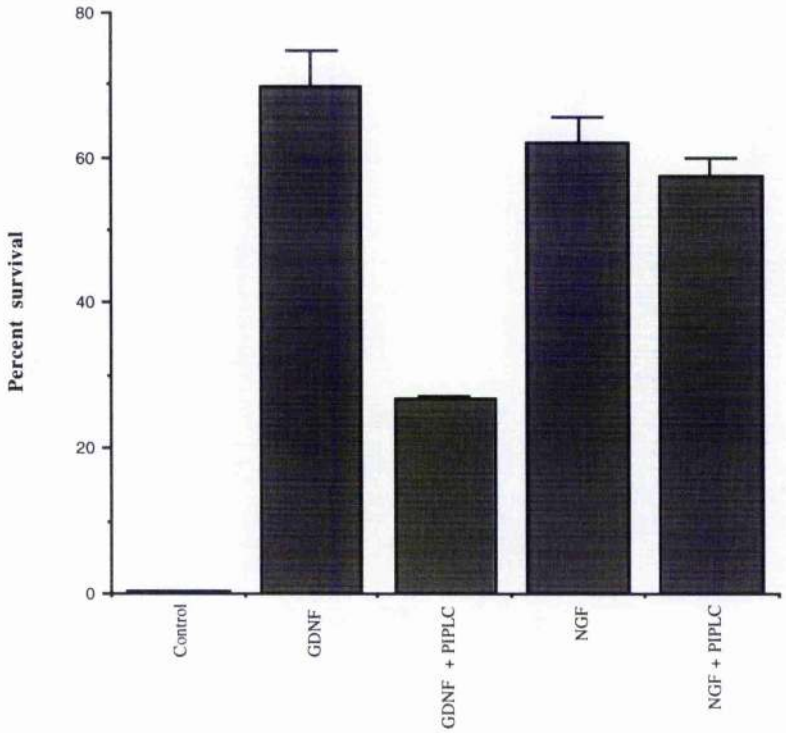


Figure 4.7. Effect of PIPLC treatment on E14 chicken sympathetic lumbar chain neurons cultured in the presence of either GDNF or NGF. Bar chart of the percent of survival of E14 chicken sympathetic lumbar chain neurons grown with either NGF (10 ng/ml) or GDNF (1 μg/ml). The means and standard errors are shown (n=4)



3.2. ECTOPIC EXPRESSION OF CHICKEN GDNFR-α AND NTNR-α ON SCG NEURONS

To ascertain the involvement of GDNFR-α and NTNR-α in the survival responses of developing neurons to their putative ligands, GDNF and neurturin, cultured neurons that do not respond to either GDNF or neurturin were microinjected to introduce expression plasmids encoding GDNFR-α and NTNR-α. Since GDNFR-α and NTNR-α are ligand binding receptors and do not tranduce the signal intracellularly, an expression vector encoding RET, the ligand transducing component of both GDNF and NTN receptor complexes, was also used for microinjection. From a survey of several different populations of neurons, sympathetic neurons of the superior cervical ganglion (SCG) of postnatal day 4 (P4) mice were found to not be supported by either GDNF or neurturin in culture. Reverse transcription/polymerase chain reaction (RT/PCR) analysis revealed that these neurons do not express endogenous mRNAs encoding GDNFR-α, NTNR-α or RET, whereas P4 mouse nodose neurons, which survive in response to GDNF and neurturin, express these mRNAs (Buj-Bello *et al.*, 1997). Furthermore, because postnatal mouse SCG neurons die rapidly in defined medium lacking neurotrophic factors, they are ideal for examining the involvement of ectopically expressed receptors in mediating neurotrophic factor responses.

3.2.1. Methods

3.2.1.a) Construction of expression vectors

GDNFR-α and NTNR-α cDNAs were subcloned into the pMEX vector. A 2.7 kb BamHI/SnaBI GDNFR-α cDNA fragment containing the full coding region was subcloned into a BamHI/SmaI linearized pMEX vector of 3.7 kb. The ligation was carried in a total volume of 11 µl, containing: 1 µl of 10x ligation buffer, 6 µl of cDNA fragment (40 ng), 1 µl of linearized pMEX (10 ng), 2 µl of distilled water and 1 µl of T4 DNA ligase (Biolabs). The mixture was incubated overnight at 4°C. The orientation of the insert was checked by restriction map analysis. A 1.8 kb NTNR-α DNA fragment containing the full coding region was subcloned into the linearized pMEX vector using the cloning sites SphI/SmaI. The 1.8 kb DNA

fragment was composed of a 110 bp PCR amplified SphI/SmaI DNA fragment (Vent DNA polymerase was used for amplification, Biolabs) and a 1758 bp SmaI/SmaI cDNA fragment. Ligations were carried out using similar conditions as those described above and insert orientations were determined by restriction map analysis. Given the fact that PCR amplification of DNA can result in mutations, the 5' end of the insert was sequenced using the termination chain reaction method (section 2.1.6). Long form RET cDNA inserted into pRc/CMV vector (a gift from A. Rosenthal) was used for microinjection.

Competent XL1-Blue E.coli cells were transformed with either the ligation products or RET plasmid, and grown in culture medium. Plasmid DNA was purified using the Qiagen mini- and midiprep kits, according to manufacturer's instructions. For microinjection, each construct was diluted at a concentration of 100 μg/ml in phosphate-buffered saline (PBS).

3.2.1.b) Neuronal culture and microinjection

Superior cervical ganglia were dissected from CD1 mice at P4. The ganglia were dissociated and the neurons were separated from non-neuronal cells by differential sedimentation (chapter 2). The resulting neuronal suspension was plated in 60 mm plastic tissue culture dishes pre-coated with poly-DL-ornithine and laminin (chapter 2). Cells were grown for 12 hours in a defined medium consisting of Ham's F14 supplemented with glutamine and 10% of SATO (containing pathocyte-4-BSA, putrescine, progesterone, L-thyroxine, sodium selenite, tri-iodo-thyronine), and 2 ng/ml of NGF. After this time, the cultures were washed extensively with neurotrophin-free medium, and neurons were pressure-injected into the nucleus (Allsopp *et al.*, 1993) with the expression plasmids (either pMEX/GDNFR-α, pMEX/NTNR-α, pRc-CMV/RET or a combination of these constructs). The cultures were supplemented with either GDNF or neurturin immediately after injection, and the number of surviving neurons was counted 48 hours later and expressed as a percentage of the number of injected neurons.

3.3.2. Results

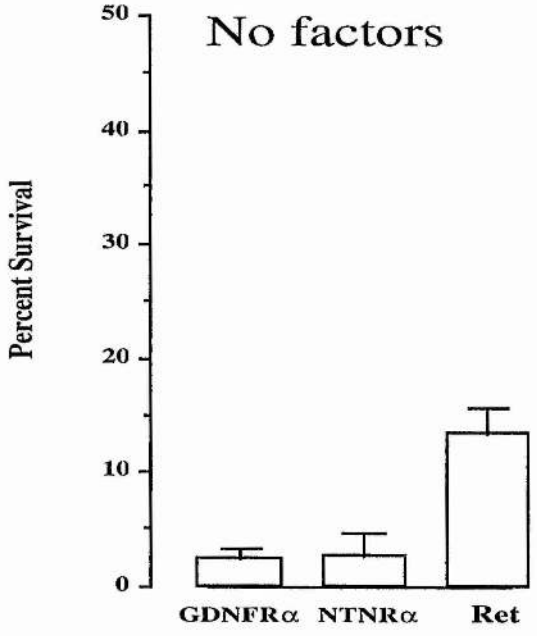
P4 mouse SCG neurons in culture were microinjected with expression plasmids encoding GDNFR-α, NTNR-α or RET. Ectopic expression of either GDNFR-α or NTNR-α alone in SCG neurons had a negligible effect on the number of neurons surviving with either GDNF or neurturin (less than 5% survived in medium containing these factors following injection with either GDNFR-α or NTNR-α expression plasmids) (figure 4.8.B). A similar low level of survival was observed in cultures of these injected neurons growing in medium without added factors (figure 4.8.A). This suggests that neither GDNFR-α nor NTNR-α alone are capable of mediating survival responses to GDNF and neurturin.

Because RET is an essential component of the GDNF receptor complex (Trupp *et al.*, 1996; Durbec *et al.*, 1996a; Jing *et al.*, 1996; Treanor *et al.*, 1996), neurons were co-injected with expression plasmids for RET and either GDNFR-α or NTNR-α to see if neurons expressing both of these receptors components would exhibit specific survival responses to GDNF and neurturin. Ectopic expression of RET alone promoted the survival of approximately 10% of SCG in the absence of added factors (figure 4.8.A). However, neither GDNF nor neurturin significantly increased the survival of RET-expressing neurons ($p > 0.05$, t-test, n=6) suggesting that RET alone is not capable of mediating survival responses to GDNF and neurturin. In contrast, neurons co-expressing NTNR-α plus RET had a substantially enhanced survival response to neurturin that was statistically greater than that of neurons expressing RET alone ($p = 0.003$, t-test, n=6). Likewise, neurons co-expressing GDNFR-α plus RET had substantially enhanced survival response to GDNF that was significantly greater than that of neurons expressing RET alone ($p = 0.0002$, t-test, $n = 8$) (figure 4.8.B). In contrast, neurons co-expressing GDNFR-α plus RET showed no enhanced survival response to neurturin and there were no more RET/GDNFR-α expressing neurons surviving with neurturin than RET-expressing neurons. Likewise, the number of RET/NTNR-α expressing neurons surviving with GDNF was not significantly different from the number of RET-expressing neurons surviving with this factor ($p > 0.2$, t-test, $n = 8$) (figure 4.8.B). These results imply that GDNFR-α and NTNR-α are receptors for GDNF and neurturin, respectively, and that RET is required for signalling and the

survival response to both factors. Although GDNFR-α and NTNR-α in the presence of RET conferred specific survival responses to GDNF and neurturin at concentrations of 5 ng/ml, at 10-fold higher concentrations of these factors some cross-talk in responsiveness was observed, with GDNF being a more promiscous neurotrophic factor than neurturin (figure 4.9).

Figure 4.8. Bar chart of the percent survival of P4 mouse SCG neurons 48 hours after microinjection with expression plasmids for GDNFR-α, NTNR-α and RET both singularly and in combination. The injected neurons were incubated in medium containing either neurturin or GDNF. The means and standard errors of the combined results of 8 separate experiments are shown (n ≥ 4 for each experimental condition).

A)



B)

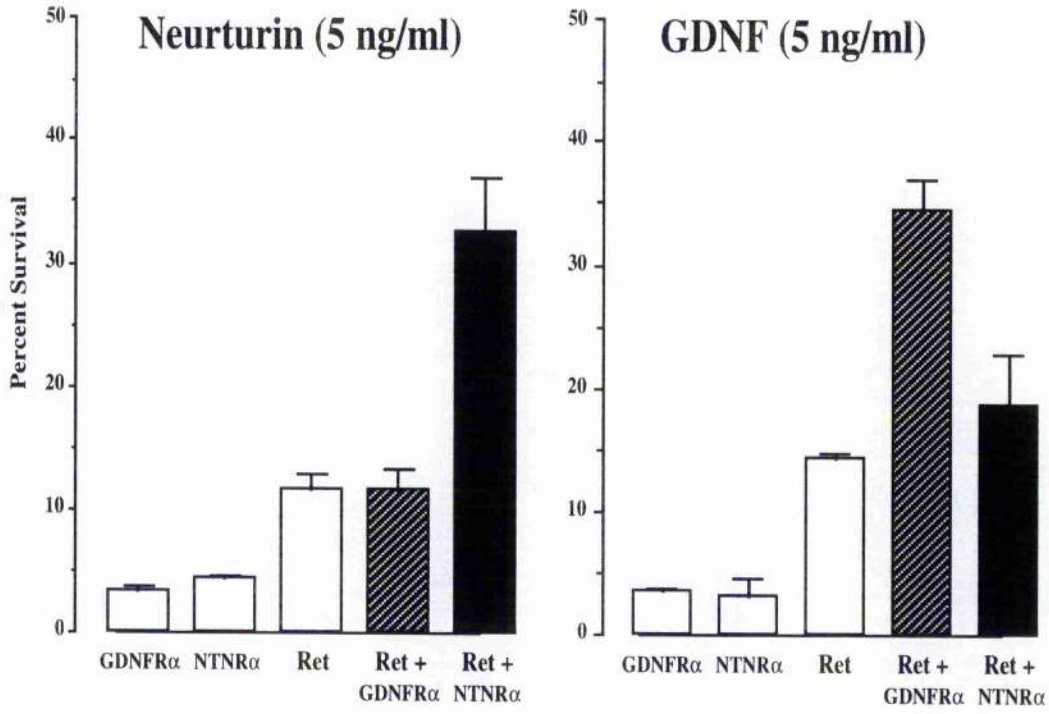
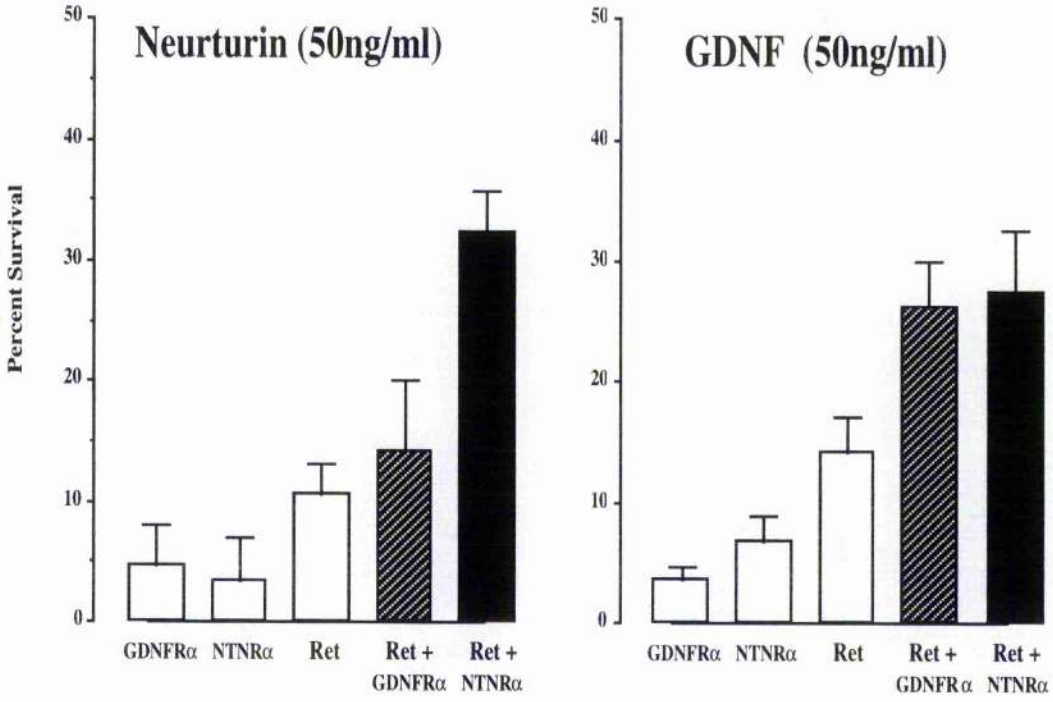

Neurturin (5 ng/ml)
GDNF (5 ng/ml)
Percent Survival
50
40
30
20
10
0
GDNFRα
NTNRα
Ret
Ret + GDNFRα
Ret + NTNRα

Figure 4.9. Bar chart of the percent survival of P4 mouse SCG neurons 48 hours after microinjection with expression plasmids for GDNFR-α, NTNR-α and RET both singularly and in combination, in the presence of 50 ng/ml of either neurturin or GDNF. The means and standard errors of the combined results of 6 separate experiments are shown (n ≥ 4 for each experimental condition).



4. EXPRESSION ANALYSIS OF CHICKEN GDNF AND NEURTURIN RECEPTORS ALPHA

4.1. EXPRESSION ANALYSIS BY NORTHERN BLOTTING

Since there is sequence homology between GDNFR-α and NTNR-α, the use of a probe from the 3' region avoids the possibility of cross-hybridization of the GDNFR-α probe with NTNR-α mRNA and *vice versa.*

4.1.1. Methods

4.1.1.a) Preparation of the probes by polymerase chain reaction amplification

Since there is sequence homology between GDNFR-α and NTNR-α, DNA probes were obtained by polymerase chain reaction (PCR) amplification of non-homologous fragments of the GDNFR-α and NTNR-α cDNA, to avoid cross-hydridisation of the GDNFR-α probe with the NTNR-α mRNA transcripts and *vice-versa*. The primers used for PCR amplification of chicken GDNFR-α and NTNR-α were: 5'-GCCACATATCCTCGGAGAATTC-3' and

5'-TAGAATGGCTTCTCAGCAGG-3' for GDNFR-α , and

5'-CCAGAAGACCTTTGTGGATCAG-3' and

5'-GACAGCCTCACACCGTTTGAAT-3' for NTNR-α.

The PCR reaction was carried out in 0.5 ml Eppendorf tubes. 36 µl of a master mixture containing 19 µl of distilled water, 2 µl of a 100 mM dNTPs, 10 µl of 25 mM $MgCl_2$ and 5 µl of 10x PCR reaction buffer (Promega) was added to 10 µl of primers (5 µl of each primer at 15 pmol/µl) and 1 µl of plasmid pBs/GDNFR-α or pBs/NTNR-α (1 ng/µl). After mixing, 1 µl of Taq DNA polymerase (Promega) was added to the tube, and after further mixing, 50 µl of mineral oil was added to avoid evaporation of the sample during PCR amplification. The PCR conditions for each of the 20 cycles were: denaturation of the double-stranded DNA for 1 minute at 95°C followed by annealing of the primers to the DNA at 60°C for 1.5 minutes and extension or DNA synthesis for 2 minutes at 72°C.

After amplification, the single PCR product was separated from the plasmid DNA and primers by electrophoresis in a 1% low-melting-temperature (LMP)

agarose gel. The amplified DNA band was cut from the gel and the DNA was purified from the agarose by STE/phenol/chloroform extraction (Sambrook *et al.*, 1989). The GDNFR-α and NTNR-α DNA fragments were then labelled by nick-translation (Sambrook *et al.*, 1989).

4.1.1.b) Northern blotting and hybridization

Several tissues were dissected from white leghorn chicken embryos at different stages of development. Total RNA was isolated, electrophoresed and blotted to Hybon-N+ membranes as previously described in chapter 2. Hybridization was also carried out according to the same procedure described in that chapter. A specific 686 bp PCR-amplified chicken GDNFR-α DNA probe, and a specific 626 bp PCR-amplified chicken NTNR-α DNA probe were used for hybridization with GDNFR-α and NTNR-α mRNAs, respectively. The probes were ^{32}P-labelled by nick-translation. Hybridization was carried out for 48 hours at 42°C in the solution described in chapter 2 and containing the radiolabelled probe. Following hybridization, the filters were washed at 65°C in 2X SSC with 0.2%SDS before exposure to Kodak Omat X-ray film. Filters were first hybridized with the ^{32}P-labelled nick-translated GDNFR-α probe. Subsequently, the DNA probe was stripped off the filters by boiling, and rehybridised with the ^{32}P-labelled nick-translated NTNR-α probe.

4.1.2. Results

Northern blotting was used to study the expression of GDNFR-α and NTNR-α transcripts in chicken embryos at different developmental stages. A single GDNFR-α transcript of approximately 10 kb and two NTNR-α transcripts of approximately 3.5 and 2.8 kb were present in the nervous system and several other tissues. However, in some tissues only one NTNR-α transcript of 3.5 kb was detected.

Whereas similar levels of NTNR-α transcripts (2.8 and 3.5 kb) were present in the spinal cord, medulla oblongata, pons, cerebellum, midbrain (including the tectal vesicles), and forebrain at E10, the expression of GDNFR-α was far higher in the spinal cord than other CNS regions at this stage (figure 4.10). Among non-neuronal tissues, Northern blotting at E10 revealed very high levels of NTNR-α

mRNA in the liver compared to other tissues such as muscle, skin, kidney, and intestine. The expression of NTNR-α mRNA was barely detectable in lung and heart at this stage of development. In non-neuronal tissues, GDNFR-α mRNA was present at moderate levels in kidney and intestine, and at low levels in liver and skin. This transcript was hardly detectable by Northern blotting in muscle, lung, and heart (figure 4.10).

Northern blotting of tissues dissected from chicken embryos at E18 revealed that both GDNFR-α and NTNR-α mRNAs were expressed at variable levels in both neuronal and non-neuronal tissues (figure 4.11). In the CNS, GDNFR-α and NTNR-α transcripts were expressed at high, moderate, and low levels in the midbrain, forebrain, and spinal cord, respectively. Among non-neuronal tissues, very high levels of NTNR-α transcripts were present in liver, whereas only very low levels of GDNFR-α mRNA were expressed in this organ. Northern blotting also revealed moderate levels of expression of NTNR-α mRNAs in the small and large intestines, and kidney, and low levels in lung, muscle, and skin. GDNFR-α transcript was present at moderate levels in lung and kidney, and at low levels in the small and large intestines, whereas it was not detected by Northern blotting in muscle and skin (figure 4.11).

Developmental changes in the expression of these transcripts were clearly observed in some tissues (figure 4.12). In the spinal cord, GDNFR-α and NTNR-α mRNA levels decreased markedly between E10 and E12 and remained at low levels at later embryonic stages. In the skin, there was a gradual decrease in the expression of GDNFR-α and NTNR-α mRNAs with age. In contrast, the expression of GDNFR-α and NTNR-α transcripts increased slightly in the midbrain and forebrain between E10 and E14.

Thus, GDNFR-α and NTNR-α mRNAs are widely distributed in neuronal and non-neuronal tissues, and the level of expression of these transcripts changes in some of tissues during the development of the chicken embryo.

Figure 4.10. Expression of GDNFR-α and NTNR-α mRNAs in the chicken embryo at E10. Northern blot of total RNA from various tissues. Filters were hybridized sequentially with GDNFR-α and NTNR-α probes. Bands corresponding to the 10 kb GDNFR-α and 3.5 and 2.8 kb NTNR-α transcripts are indicated. Ethidium bromide picture of the 28S ribosomal RNA on filters is shown to compare the relative levels of total RNA present in each sample.

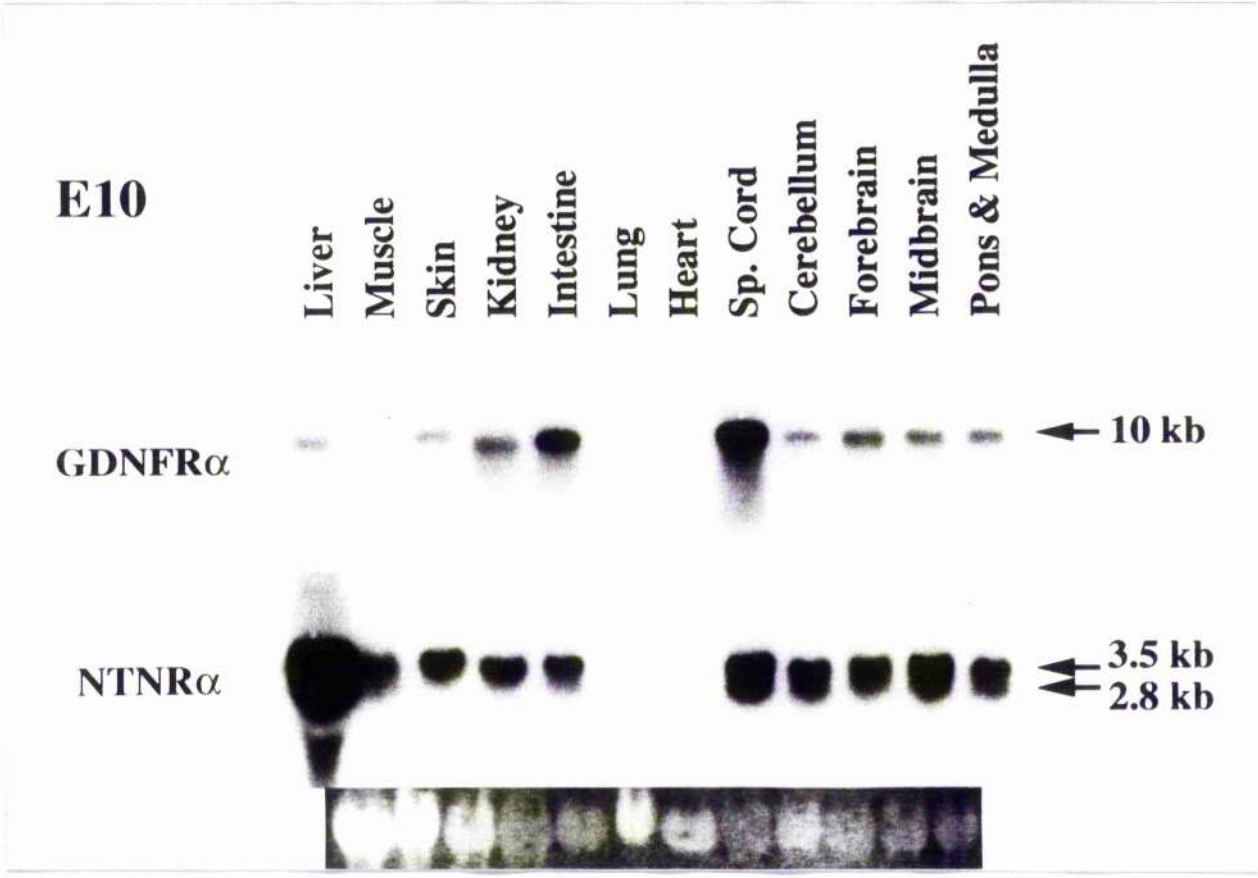


Figure 4.11. Expression of GDNFR-α and NTNR-α mRNAs in the chicken embryo at E18. Northern blot of total RNA from various tissues of chicken embryos at E18. Filters were hybridized sequentially with GDNFR-α and NTNR-α probes. Bands corresponding to the 10 kb GDNFR-α and 3.5 and 2.8 kb NTNR-α transcripts are indicated. Ethidium bromide staining of the 28S ribosomal RNA on filters is shown to compare the relative levels of total RNA present in each sample.

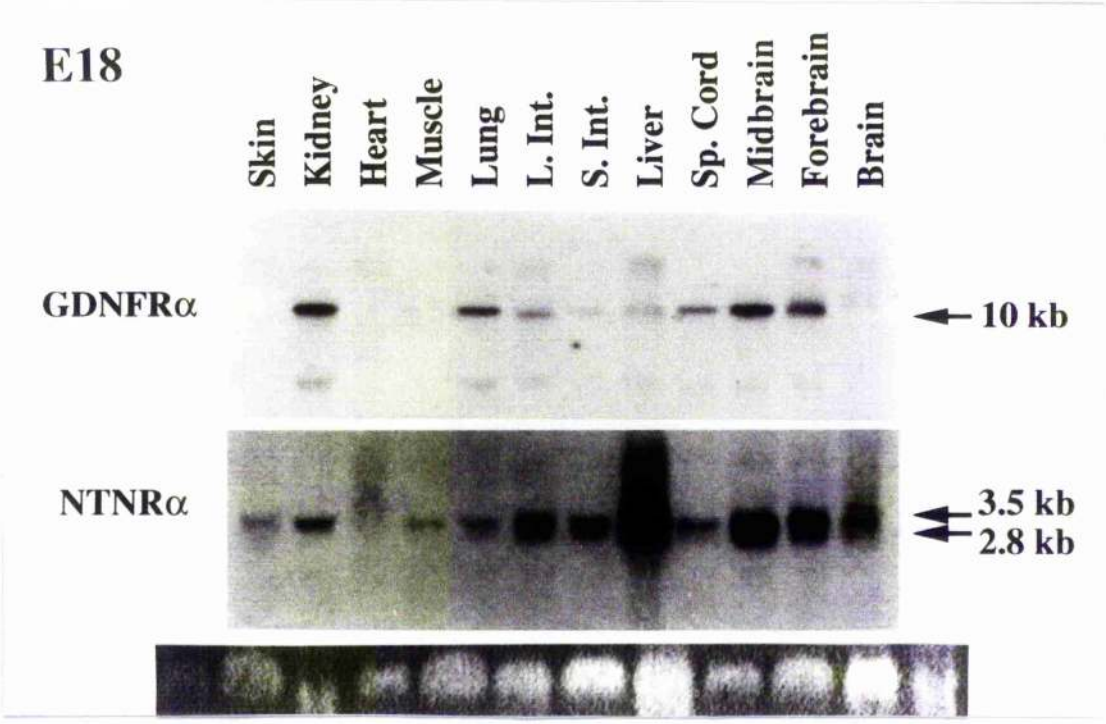
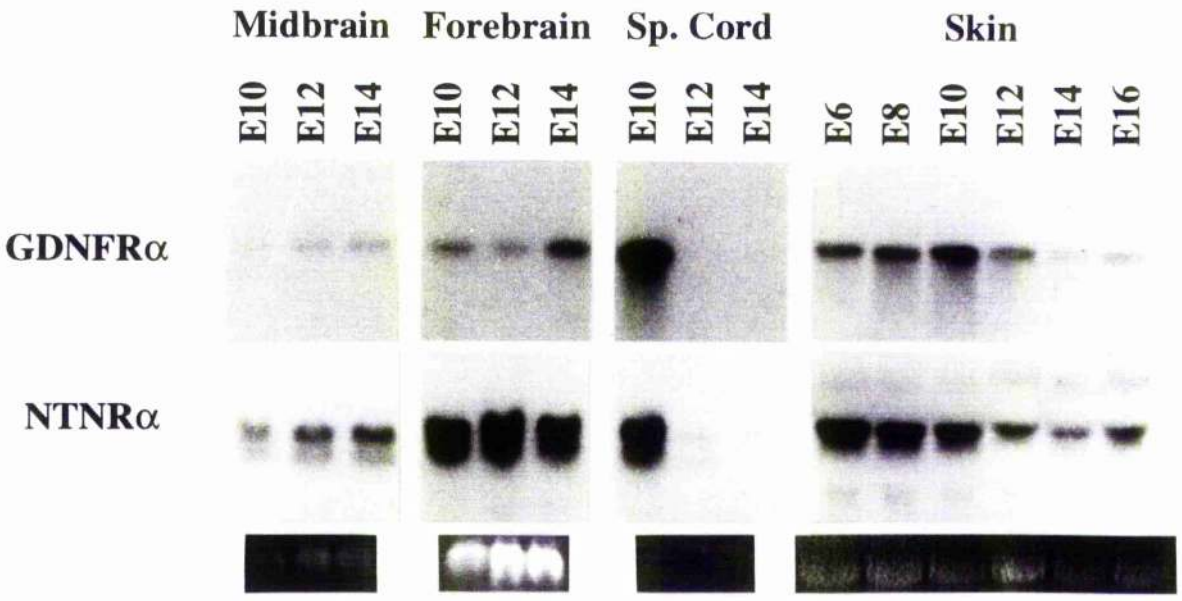


Figure 4.12. Developmental changes in the expression of GDNFR-α and NTNR-α mRNAs in the chicken embryo. Northern blot of total RNA from chicken midbrain, forebrain, spinal cord, and skin at different ages. Filters were hybridized sequentially with GDNFR-α and NTNR-α probes. Bands corresponding to GDNFR-α and NTNR-α transcripts are shown. In the photograph of the ethidium bromide stained gel (midbrain, forebrain and spinal cord) and filter (skin), 28S ribosomal RNA is shown to compare the relative levels of total RNA present in each sample.



4.2. EXPRESSION ANALYSIS BY *IN SITU* HYBRIDIZATION

4.2.1. Methods

4.2.1.a) Cryosectioning of chicken embryos

Chicken brain and trunk from embryonic days 10 and 18 were dissected, embedded in OCT component (Tissue Tek), and immediately frozen in liquid nitrogen. Serial sections between 15 µm and 30 µm were cut using a cryostat and mounted on poly-L-lysine-coated slides. Sections were kept at -80°C until use.

4.2.1.b) Synthesis of digoxigenin-labelled riboprobes

The synthesis of digoxigenin (DIG)-labelled riboprobes involves several steps.

4.2.1.b)1. Subcloning of DNA fragments into a vector

The 686 and 626 bp PCR-amplified DNA fragments of GDNFR-α and NTNR-α used as probes for hybridization of Northern blots (this chapter) were subcloned into the vector pGEM-T (Promega). For the ligation reaction, 12 ng and 6 ng of vector DNA was mixed with 50 ng and 25 ng of 686 and 626 bp DNA fragments, respectively, in Eppendorf tubes. 1 µl of 10x ligation buffer (Promega) was added to each tube and the final volume was made up to 10 µl with distilled water. 1 µl of T4 DNA ligase (3 units/µl, Promega) was added and the mixture was incubated for 4 hours at 4°C for the ligation reaction to proceed. XL1 Blue competent E.coli bacteria were transformed with the product of the ligation reaction. Following transformation, restriction analysis of miniprep DNA was used to select bacterial colonies containing plasmid vectors carrying the correct insert DNA and to determine the orientation of the insert. Correctly identified colonies were then grown up to amplify and isolate plasmid DNA (for procedures, see Sambrook *et al.*, 1989; this chapter).

4.2.1.b)2. Linearization of plasmids

The plasmid pGEM-686bp insert was linearized by digestion with the endonucleases Sac II and Pst I, whereas the plasmid pGEM-626bp insert was

linearized by digestion with the restriction enzymes Sal I and Sph I, for reasons given below. Fully digested plasmids were purified by phenol/chloroform extraction (Sambrook *et al.*, 1989).

4.2.1.b)3. Synthesis of sense and antisense DIG-labelled RNA

The following solutions were added and mixed in an Eppendorf tube: 4 µl of 5x transcription buffer (DIG RNA labelling kit, Boehringer), 2 µl of 0.1 M DTT, 2 µl of 10x DIG RNA labelling mix (Boehringer), 1 µl (38 units) of ribonuclease inhibitor (RNA Guard, Pharmacia), 10 µl (150 ng) of linearized plasmid, 1 µl of either T7 RNA polymerase (50 units, Gibco) or SP6 RNA polymerase (20 units, Promega). The mixture was incubated for 1.5 hours at 37°C. After one hour of incubation the RNA synthesis was monitored by loading a sample of the reaction in an agarose gel. For synthesizing GDNFR-α sense and antisense RNA, the plasmid pGEM-686bp insert was linearized with Sac II and Pst I, respectively, and the transcription enzyme used was SP6 and T7 RNA polymerase, respectively. In the case of NTNR-α sense and antisense RNA synthesis, the Sal I linearized pGEM-626bp plasmid and the SphI linearized pGEM-626bp plasmid were incubated with T7 and SP6 RNA polymerase, respectively.

The transcription reaction was stopped by addition of 130 µl of STE and 5 µl of 10% SDS. Sephadex G-50 column (Nick Column, Pharmacia Biotech) was used according to manufacturer's instructions to separate the DIG-labelled riboprobe from the unincorporated nucleotides. Prior to use, the Sephadex G-50 column was washed with STE containing 0.1% SDS. Following this, 50 µl of tRNA (10 mg/ml) was added to the column to avoid non-specific binding of the synthesized RNA to the Sephadex. Finally, the column was washed again with STE containing 0.1% SDS. The RNA was precipitated by the addition of 1:20 vol. of 3 M sodium acetate and 2-2.5 vol. ethanol followed by overnight incubation at -20°C. Subsequently, the RNA pellet was washed in 70% ethanol and resuspended in 50 µl of distilled water. Approximately 1 µg of RNA was synthesized and recovered from each transcription reaction using this procedure.

The synthesized RNA (1 µg) was finally diluted in 1 ml of *in situ* hybridization buffer. 50 ml of this buffer contained the following reagents: 25 ml of formamide, 12.5 ml of 20x SSC, 0.5 ml of 10% Triton X-100, 2.5 ml of CHAPS,

5 ml of yeast RNA (10 mg/ml), 0.25 ml of 1 M EDTA pH 8.0, 1 ml of distilled water, 2.5 ml of Heparin (1 mg/ml), 0.75 ml of 100x Denhardt's solution, and 1 gr of Blocking reagent (Nucleic Acid Detection Kit, Boehringer).

4.2.1.c) *In situ* hybridization

4.2.1.c)1. Pre-treatment of sections for hybridization

The slides containing the sections were kept in boxes at -80°C before use. Prior to hybridization, the slides were placed in racks and immersed for 5 minutes in chilled 4% paraformaldehyde/PBS, pH 7.5. Following this, the sections were rinsed twice in PBS, pH 7.5, before being immersed for 10 minutes in 0.1 M triethanolamine pH 8.0/ PBS + (v/v) acetic anhydride 0.25%. Next, the slides were immersed twice, for 2 minutes each time, in PBS pH 7.5 followed by 2 minutes in each of 70% ethanol, 96% ethanol and 100% ethanol. After a 5 minutes soak in chloroform and 2 minutes in 100% ethanol, followed by 2 minutes in 96% ethanol, the sections were finally air dried in a laminar flow hood.

4.2.1.c)2. Hybridization

A few drops of either sense or antisense RNA (1 µg/ml in hybridization buffer) were distributed on the surface of each section. The sections were then covered by a piece of parafilm to avoid evaporation of the solution, and placed into a humified box. Hybridization was carried out in a water bath, for 24 hours, at 55°C.

4.2.1.c)3. Post-hybridization washes

After hybridization, the sections were washed sequentially for 5-10 minutes, at 55°C, in the following solutions: solution I (50% formamide, 5x SSC, 0.1% Triton X-100, 0.5% CHAPS); 75% solution I/2x SSC; 50% solution I/2xSSC; 25% solution I/2x SSC; twice in 2x SSC/0.1% CHAPS; 0.2x SSC/0.1% CHAPS. Then, the sections were washed twice for 5-10 minutes at room temperature in TBT (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.1% Triton X-100). This was followed by a 30 minutes room temperature wash in 1% Blocking agent/TBT with 2% sheep serum and two 5 minutes washes in TBT.

4.2.1.c)4. Incubation with anti-digoxigenin antibodies

100-150 µl of 1:150 dilution of anti-digoxigenin-AP, Fab fragment (Boehringer) in TBT/2% sheep serum was distributed on the surface of the sections, and incubated overnight at 4°C.

4.2.1.c)5. Post-antibody washes

After incubation with antibodies, the sections were washed three times at room temperature, for 10 minutes, in TBT, followed by three washes for 5 minutes in NTM (100 mM NaCl, 100 mM Tris-Cl pH 9.5, 50 mM $MgCl_2$, 0.1% Tween-20). Finally, the sections were incubated at 4°C in 4.5 µl of NBT solution (Boehringer) + 3.5 µl of bovine calf intestine phosphatase (Cip)/ml of NTM, and left in a dark place for few hours until the colorimetric reaction had finished. The extend of the colorimetric reaction was monitored using a microscope. The slides were then mounted with AF1, a glycerol/PBS mounting solution (Citifluor), and kept at 4°C until examination under the microscope.

4.2.2. Results

In situ hybridization was used to analyse the expression at the cellular level of both GDNFR-α and NTNR-α mRNAs in neural tissues. This technique is especially useful for detecting the presence or absence of these transcripts in the ganglia of the peripheral nervous system, bearing in mind that neurons from most ganglia respond *in vitro* to both GDNF and neurturin.

In accordance with Northern blot analysis, *in situ* hybridization revealed prominent expression of GDNFR-α and NTNR-α in spinal motoneurons at E10 and low levels of both transcripts in these neurons at E18 (figure 4.13). In the peripheral nervous system, GDNFR-α and NTNR-α were prominently expressed in the ganglia of the sympathetic lumbar chain and dorsal root ganglia (figure 4.13). *In situ* hybridization also revealed marked expression of GDNFR-α mRNA in cerebellar granule cells (GC), Purkinje cells (PC), and neurons in the deep cerebellar nuclei (DN) (figure 4.14). The same pattern of expression was observed for NTNR-α mRNA in cerebellum (not shown). When using DIG-labelled sense riboprobes as a negative control for hybridization, the probes did not hybridize with any structure, and only a general low background was detected (not shown).

Figure 4.13. Transverse sections of the lumbar region of E10 and E18 chicken embryos hybridized with DIG-labelled GDNFR-α and NTNR-α antisense riboprobes. Localisation of a dorsal root (DRG) and sympathetic chain ganglion (SC) is shown. Motoneurons in the ventral spinal cord are indicated with an arrow. Scale bar = 200 μm.

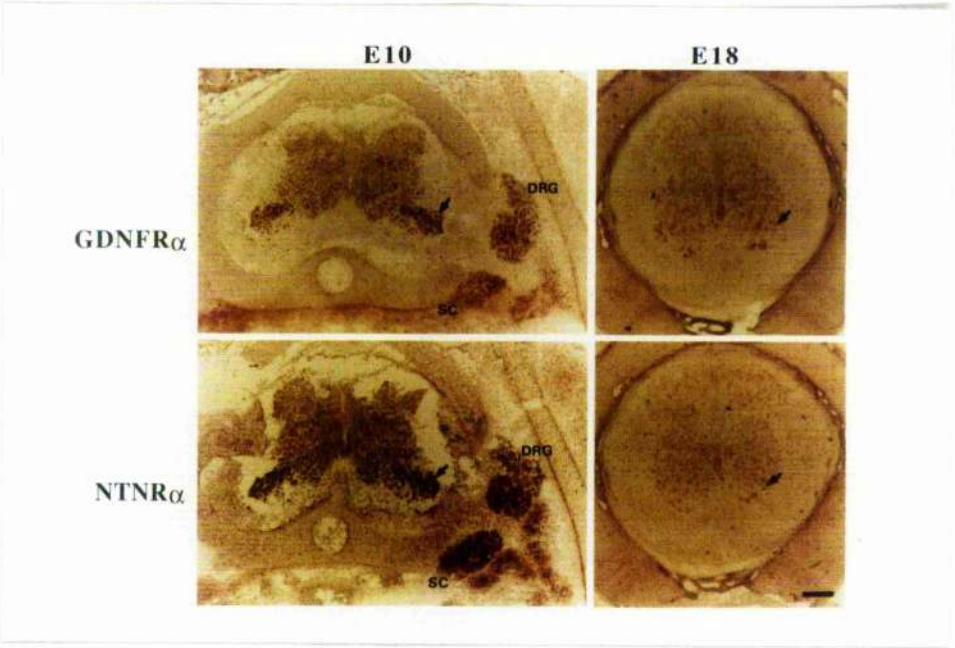
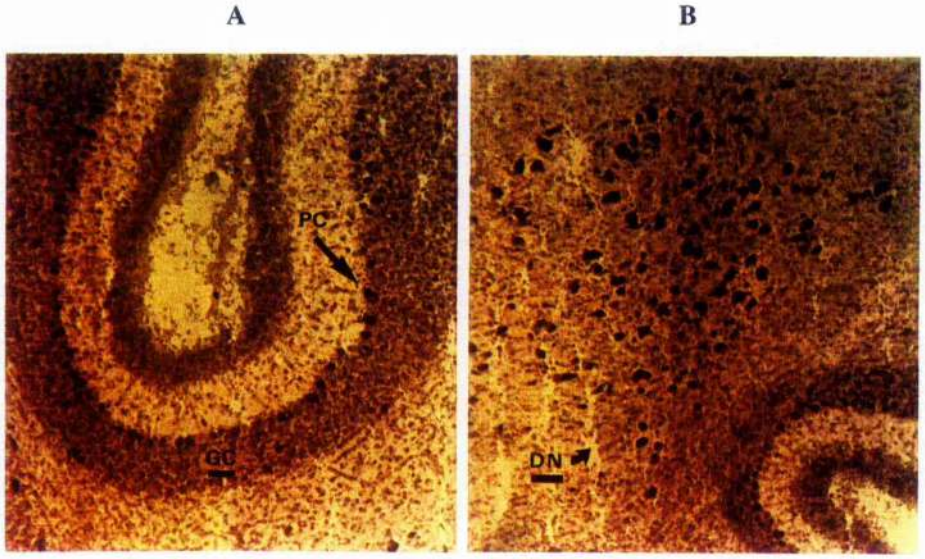


Figure 4.14. Sagital section of the cerebellum of E18 chicken embryo hybridised with DIG-labelled GDNFR-α antisense riboprobe. Localisation of Purkinje cells (PC), granule cells (GC), and neurons from the deep nuclei (DN) is shown.



4.3. EXPRESSION ANALYSIS BY RT-PCR

The aim of this study was to analyse the expression of GDNFR-α and NTNR-α mRNAs in embryonic chicken PNS neurons that respond to both GDNF and neurturin *in vitro*. Since the number of neurons obtained from each dissection is too small for Northern blot analysis, specific mRNAs were detected by PCR amplifying reverse transcribed total RNA (RT-PCR). A semiquantitative RT-PCR assay was used to compare the relative levels of GDNFR-α and NTNR-α transcripts in certains populations of PNS neurons at E10 and E14.

4.3.1. Methods

4.3.1.a) Dissection and purification of neurons

E10 ciliary, nodose, and sympathetic lumbar chain ganglia, and E14 dorsomedial trigeminal ganglion (DMTG) were dissected from White Leghorn chicken embryos. Neurons were separated from non-neuronal cells by differential sedimentation (Davies, 1988a). Purified neurons were immediately frozen and kept at -80°C until further use.

4.3.1.b) RNA extraction for PCR

Total RNA was extracted from neurons that were purified from 30 to 50 ganglia. Cells were lysed in 0.5 ml of solution D (for composition, see chapter 2) by passing the neuronal suspension through a 25 gauge needle fitted to a 1 ml syringe. Eppendorf tubes containing the cell lysates were placed on ice, and the following solutions were added sequentially: 2 µl of E.coli tRNA (5 mg/ml), 50 µl of 2 M sodium acetate pH 4.4, 0.5 ml of water saturated acidic phenol, and 150 µl of 25:1 chloroform:isoamyl alcohol. The mixture was shaken vigorously, incubated on ice for 15 minutes, and centrifuged at 13,000 rpm for 30 minutes. The upper aqueous phase was transferred to fresh tubes, and RNA was precipitated by the addition of two volumes of ethanol followed by incubation at -20°C overnight. Tubes were then centrifuged at 13,000 rpm for 30 minutes. The RNA pellet was washed in 70% ethanol and air dried on the bench. The RNA was resuspended in 50 µl of 10 mM Tris pH 7.5, 6 mM $MgCl_2$, 20 mM VRC's (vanadyl-ribonucleoside

complex, Sigma), 2.5 units of RNase-free DNase I (Pharmacia). The mixture was incubated at 37°C for 1 hour. The RNA was further purified using an RNaid kit (BIO 101). In brief, three volumes (150 µl) of $NaClO_3$ and 30 µl of RNA binding matrix were added to the RNA followed by incubation for 5-10 minutes at room temperature to allow RNA to bind to the matrix. The matrix was sedimented by brief centrifugation and washed twice in 0.7 ml of RNaid wash solution. The remaining RNAid wash solution was completely removed, with a fine pipette tip, and RNA was eluted from the matrix by resuspension in 100 µl of distilled water and incubation at 60°C for 3 minutes. Following centrifugation, at 13,000 rpm for 1 minute, the supernatant containing the RNA was tranferred to fresh tubes and stored at -80°C until use.

4.3.1.c) Reverse transcription

For each reaction, 2 µl of resuspended RNA was reverse transcribed in a 40 µl final volume reaction containing: 8 µl of 5x RT Superscript buffer, 4 µl of 5 mM dNTPs, 4 µl of 100 µM random hexanucleotides (RH), 4 µl of 0.1 M DTT. For negative controls, RNA was replaced by distilled water. The sample was heated for 3 minutes at 90°C, and then 1 µl of Superscript reverse transcriptase (Gibco) was added to the mixture. Another negative control with no reverse transcriptase was performed. The reaction was allowed to proceed for 1 hour at 37°C, after which time it was stopped by heating the sample for 6 minutes at 95°C.

4.3.1.d) Polymerase chain reaction

Chicken GDNFR-α, NTNR-α and GAPDH cDNAs were amplifified by PCR from cDNA that had been synthesized from RNA extracted from E10 ciliary, sympathetic lumbar chain, and nodose ganglion neurons, and E14 DMTG neurons. For each PCR reaction, 35 µl of a master mix solution was added to an 0.5 ml Eppendorf tube. The composition of the master mix (n, number of PCR) was: (nx) 5 µl of 10x PCR buffer (Promega), (nx) 10 µl of $MgCl_2$, (nx) 2 µl of 100 mM dNTPs, and (nx) 18 µl of distilled water. The following solutions were added to each tube containing 35 µl of master mix: 5 µl of each primer, 5 µl of cDNA, 1 µl of Taq DNA polymerase (Promega). After mixing, each reaction was overlayed with 50 µl of mineral oil. The amounts of cDNA in samples were normalised by

amplification of a fragment of GAPDH cDNA.

The primers used were:

Chicken GAPDH: 5'-TGGTGTCTTCACCACCAT-3' and
5'-GCATGGACAGTGGTCATA-3'

Chicken GDNFR-α: 5'-GCCACATATCCTCGGAGAATTC-3' and
5'-TAGAATGGCTTCTCAGCAGG-3'

Chicken NTNR-α: 5'-CCAGAAGACCTTTGTGGATCAG-3' and
5'-GACAGCCTCACACCGTTTGAAT-3'

The tubes were transferred to the heating block of a PCR machine and amplification was carried out for 35 cycles using the following parameters: denaturation at 95°C for 1 minute, annealing at 60°C for 1.5 minutes, and DNA synthesis at 72°C for 2 minutes.

The PCR products of each reaction were then electrophoresed in a 1% agarose gel. Bands of 686, 626, and 242 bp were present corresponding to amplified GDNFR-α, NTNR-α, and GAPDH DNA fragments, respectively.

4.3.2. Results

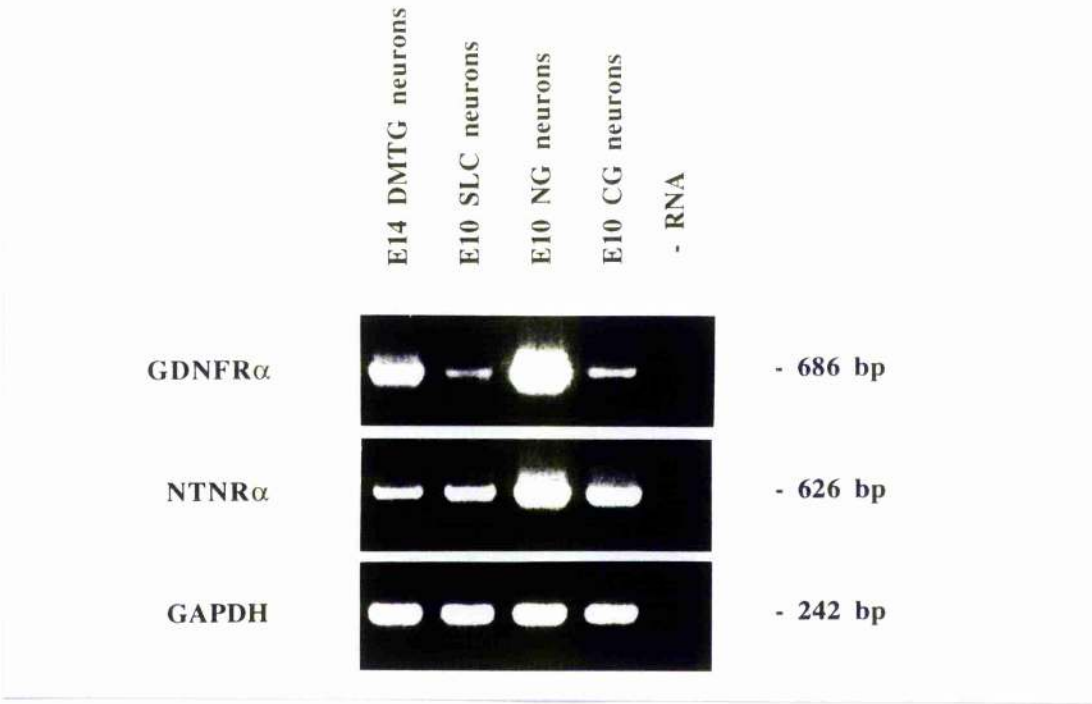
Chicken E10 sympathetic, nodose and ciliary ganglion neurons, and E14 DMTG neurons are supported by GDNF and neurturin in culture (chapters 2 and 3 of this thesis). Furthermore, the chicken GDNFR-α and NTNR-α have been cloned and demonstrated to be the ligand-specific components of the receptor complex that mediates GDNF and neurturin signalling in neurons, respectively (this chapter). By using RT-PCR of total RNA isolated from these neurons, it was demonstrated that both GDNFR-α and NTNR-α mRNAs are expressed by these cell types (figure 4.15). The level of expression of GDNFR-α mRNA was similar in E10 sympathetic and parasympathetic neurons, but higher in both E10 enteroceptive and E14 small-cutaneous sensory neurons. In contrast, NTNR-α mRNA was present at similar levels in all of these neuronal types, although a moderately higher level was observed in nodose ganglion neurons (figure 4.15).

When comparing the level of expression of both GDNFR-α and NTNR-α transcripts in each population of neurons, it was observed that in some kinds of neurons, like ciliary ganglion and sympathetic chain neurons, NTNR-α mRNA was

more highly expressed than GDNFR-α mRNA. In contrast, some other populations of neurons, like nodose ganglion and DMTG neurons, expressed more GDNFR-α than NTNR-α mRNAs (figure 4.15).

These findings demonstrate that GDNF and neurturin responsive PNS neurons express *in vivo* GDNFR-α and NTNR-α mRNAs.

Figure 4.15. RT-PCR detection of endogenous GDNFR-α and NTNR-α transcripts in chicken ciliary, nodose, dorsomedial trigeminal ganglion, and sympathetic lumbar chain neurons. The presence of similar amounts of total RNA in samples is demonstrated by amplification of a 242 bp fragment of GAPDH mRNA.



V. DISCUSSION

The results presented in this chapter report the cloning and characterization of the chicken receptors for GDNF and neurturin. The chicken receptor for GDNF is homologous to the recently discovered rat GDNFR-α (Jing *et al.*, 1996; Treanor *et al.*, 1996). The chicken receptor for neurturin, termed NTNR-α, is a novel protein that is structurally related to GDNFR-α. The cloning of rat, mouse and human homologues of NTNR-α has also been reported recently (Klein *et al.*, 1997; Baloh *et al.*, 1997). It is shown that both chicken GDNFR-α and NTNR-α are GPI-linked cell-surface proteins that lack a cytoplasmic domain capable of transducing the extracellular signal to the intracellular compartment. Furthermore, it is demonstrated by microinjection that GDNFR-α and NTNR-α play a key role in ligand discrimination in the physiological responses of neurons to GDNF and neurturin, and that RET receptor tyrosine kinase is a common transmembrane component of the active GDNF and neurturin receptor complexes that transduces the ligand signal into the cytoplasm. The physiological relevance of GDNFR-α and NTNR-α during embryonic development is shown by analysis of their expression pattern. The transcripts for these receptors are widely distributed in the nervous system and in non-neuronal tissues. Significantly, these transcripts are expressed in GDNF- and neurturin-responsive neurons from the peripheral nervous system.

In agreement with the protein structure of both chicken GDNFR-α and NTNR-α described in this chapter, human and rodent GDNFR-α and NTNR-α protein sequences have also been reported and display similar characteristics to their chicken homologue. Thus, both avian and mammalian receptors are small proteins that contain an amino-terminal hydrophobic putative signal sequence for secretion, a short carboxy-terminal hydrophobic region, a cleavage/binding consensus sequence for GPI-linkage and 30 conserved cysteine residues (Jing *et al.*, 1996; Treanor *et al.*, 1996; Klein *et al.*, 1997; Baloh *et al.*, 1997). Furthermore, chicken GDNFR-α is 80% identical to rat GDNFR-α at the amino acid level, and the sequence identity between GDNFR-α and NTNR-α, which is 49% in the chicken, is 48% in human (Klein *et al.*, 1997; Baloh *et al.*, 1997). Thus, GDNFR-α and NTNR-α constitute a novel family of structurally related receptors. It is likely that other members of the

family exist and their identification forms the basis of future research.

Several isoforms of TrkA, TrkB and TrkC containing truncations in either their intracellular or extracellular domains have previously been identified (Klein *et al.*, 1990; Middlemas *et al.*, 1991; Barker *et al.*, 1993; Valenzuela *et al.*, 1993; Tsoulfas *et al.*, 1993; Ninkina *et al.*, 1997). It would be interesting to analyse whether truncated variants of GDNFR-α and NTNR-α also exist, because this could affect either the binding to their ligands or their interaction with RET. In fact, a truncated isoform of NTNR-α with a deletion of 133 amino acids near the amino terminal region has already been identified, however, it has not been shown whether the physical interaction of this variant with other proteins is altered by the deletion (Baloh *et al.*, 1997).

The results in this chapter show that chicken neurons that respond to GDNF and neurturin loose responsiveness to these factors, but not to neurotrophins, when cultured in the presence of PIPLC, an enzyme that specifically hydrolyses GPI moieties. Similarly, the number of cultured rat spinal motoneurons surviving with either GDNF or neurturin is greatly reduced following PIPLC treatment (Treanor *et al.*, 1996; Klein *et al.*, 1997). This indicates that GDNF and neurturin survival responses in neurons are mediated via a GPI-linked receptor. Most convincingly, the survival response of these PIPLC-treated neurons to either GDNF or neurturin can be restored by administration of a soluble form of GDNFR-α and NTNR-α, respectively (Treanor *et al.*, 1996; Klein *et al.*, 1997). Furthermore, the neurturin-induced survival of P4 SCG neurons that ectopically co-express NTNR-α and RET is markedly reduced by PIPLC treatment (Buj-Bello *et al.*, 1997). In agreement with the results obtained from primary neuronal cultures, GDNF administered with soluble GDNFR-α induces RET autophosphorylation in Neuro-2A cells, a neuroblastoma cell line that expresses intrinsically high levels of RET, and PIPLC treatment of these tumoral cells expressing ectopically GDNFR-α decreases the binding of GDNF to the cell surface (Jing *et al.*, 1996). Thus, it seems that both GDNFR-α and NTNR-α are receptors bound to the membrane via a GPI linkage. GDNF and NTN appear to be members of an emerging class of neurotrophic factors that signal through a receptor complex that includes a GPI-linked component, with CNTF being the founder of this class. The CNTF receptor is a tripartite receptor

that also contains an alpha component (Davis *et al.*, 1991). CNTFRα is suficient to confer CNTF responsiveness upon transfection in cells that respond to LIF and express both gp130 and LIFRß, the other two components of the CNTF receptor (Ip *et al.*, 1993c). Furthermore, cell lines not normally responsive to CNTF respond to treatment with a combination of CNTF and soluble form of CNTFRα (Davis *et al.*, 1993b). Although funcionally similar, CNTFRα is not structurally related to either GDNFR-α or NTNR-α.

The current model for GDNF and neurturin receptors implies GDNFR-α and NTNR-α as the ligand-binding GPI-linked proteins in multicomponent receptors that contain RET protein tyrosine kinase as a common transmembrane signalling component. Results in this chapter support this model, since ectopic expression of either GDNFR-α or NTNR-α alone in mouse P4 SCG neurons do not confer responsiveness to GDNF and neurturin, respectively. Similarly, neither GDNF nor neurturin promotes the survival of neurons that ectopically express RET alone. In contrast, co-expression of either GDNFR-α plus RET or NTNR-α plus RET induces survival responsiveness to GDNF and neurturin, respectively, of intrinsically non-responsive neurons. Thus, microinjection has proven to be a powerful tool for studying the function and binding abilities of receptors into a physiological context. In agreement with this model, GDNFR-α was the only receptor isolated by expression cloning strategies searching for GDNF binding proteins (Jing *et al.*, 1996; Treanor *et al.*, 1996), and both GDNF and neurturin can induce RET tyrosine phosphorylation (Trupp *et al.*, 1996; Durbec *et al.*, 1996a; Jing *et al.*, 1996; Treanor *et al.*, 1996; Vega *et al.*, 1996; Worby *et al.*, 1996; Klein *et al.*, 1997; Baloh *et al.*, 1997).

There is still controversy as to whether GDNF and neurturin bind directly to RET or whether they interact first with GDNFR-α and NTNR-α, respectively, and then this complex associates with RET. Transfection studies on cell lines have demonstrated that GDNF can stimulate tyrosine phosphorylation of RET when GDNFR-α is present, but not when RET is expressed alone, and that RET is co-immunoprecipitated with GDNF antibodies in cells that also express GDNFR-α (Jing *et al.*, 1996; Treanor *et al.*, 1996). Similarly, neurturin is able to induce RET phosphorylation only when NTNR-α is present (Baloh *et al.*, 1997), and NTNR-α

co-immunoprecipitates with RET after neurturin exposure in cells ectopically expressing both RET and NTNR-α (Klein *et al.*, 1997). However, in apparent contrast to these data, RET has been immunoprecipitated with anti-GDNF antibodies and induced to autophosphorylate after GDNF treatment in MN1 cells, a motoneuron hybrid cell line that responds to GDNF and expresses RET (Trupp *et al.*, 1996). Furthermore, iodinated GDNF can be co-immunoprecipitated with anti-RET antibodies in RET transfected, but not in untransfected, 3T3 fibroblasts, and in LA-N-5 cells that express high endogenous levels of RET mRNA. Moreover, a low affinity binding of GDNF to RET expressing cells has been detected (Trupp *et al.*, 1996; Vega *et al.*, 1996). Although it is not clear whether RET binds directly to GDNF, it has been demonstrated that RET is an essential component of the receptor complex. Accordingly, GDNF-induced neuronal outgrowth of neuroblasts is severely impaired in RET deficient mice (Durbec *et al.*, 1996a). Nevertheless, it will be important to determine whether GDNFR-α mRNA (or related proteins, like NTNR-α) is expressed in the cell lines used for these controversial experiments. Alternatively, the generation of mice models lacking the GDNFR-α and/or the NTNR-α genes will clarify if these receptors are physiologically necessary for GDNF and neurturin signal. It would be also interesting to see if PNS neurons from RET deficient mice respond to either GDNF or neurturin in culture, and compare these results with those obtained from either GDNFR-α or NTNR-α knockout mice and wild type mice. Furthermore, this could indicate if RET is the only transmembrane receptor involved in transducing GDNF and neurturin signalling.

The results presented in this chapter show that RET is a signal transducing component shared between GDNF and neurturin, and that the ligand specificity is conferred by the alpha component of their receptor complexes. However, it is also demonstrated that GDNF and neurturin, at high concentrations, can interact with NTNR-α and GDNFR-α, respectively. Accordingly, P4 SCG neurons co-expressing either NTNR-α and RET or GDNFR-α and RET can survive in the presence of GDNF and neurturin, respectively. These results are in agreement with data reported recently that suggest a low-affinity interaction between neurturin and GDNFR-α and between GDNF and NTNR-α with, in both cases, a K_d > 1nM (Klein

et al., 1997). Furthermore, in NTNR-α (also named TrnR2, TGF-ß related neurotrophic factor receptor 2) and RET co-expressing fibroblasts, neurturin and GDNF both stimulate RET phosphorylation in a dose-dependent manner, although with neurturin being 30 times more potent than GDNF (Baloh *et al.*, 1997). Thus, it appears as if GDNF and neurturin would interact at high affinity with GDNFR-α and NTNR-α, respectively, and some cross-reactivity is observed when the receptors are exposed to high concentrations of the heterologous ligands. Surprisingly, RET phosphorylation in fibroblasts that ectopically co-express GDNFR-α (or TrnR1) and RET is induced by neurturin and GDNF with the same potency, suggesting that GDNFR-α interacts with the same affinity with GDNF and neurturin (Baloh *et al.*, 1997). Taken together, these data generate a new controversy about the specificity of GDNFR-α and NTNR-α for their ligands that remains to be clarified. If cross-reactivity exists physiologically, then the terms GDNFR-α and NTNR-α will have to be changed to TrnR1 and TrnR2, respectively, as already proposed by Baloh *et al.* Null GDNFR-α and NTNR-α mutant mice may be useful for analysing the existence of cross-reactivity. For example, it would be interesting to study the *in vitro* response of PNS neurons from NTNR-α and GDNFR-α knockout mice to GDNF and neurturin, respectively.

Northern blotting of total RNA from several chicken tissues has revealed that GDNFR-α and NTNR-α mRNAs are often co-expressed and both are widely distributed in neuronal and non-neuronal tissues. Chicken GDNFR-α is expressed as a single 10 kb transcript, whereas two NTNR-α mRNAs of 3.5 kb and 2.8 kb are found in most tissues. Similarly, two NTNR-α messages of approximately 4 kb have been detected in adult mouse brain (Baloh *et al.*, 1997). In the chicken central nervous system, GDNFR-α and NTNR-α mRNAs are found in all regions analysed, including midbrain, forebrain and spinal cord at both E10 and E18, and cerebellum and pons-medulla at E10. Interestingly, the level of expression of these transcripts in spinal cord at E10 is relatively high compared to other regions of the CNS, but it is relatively low at E18. Accordingly, GDNFR-α and NTNR-α expression in the chicken spinal cord drops markedly from E10 to E12, and remains low at later ages. *In situ* hybridization has revealed that these transcripts are localised in the ventrolateral cell columns of the chicken spinal cord, and supports the observation

that expression is higher at E10 than at E18. Similarly, the level of expression of GDNFR-α in the ventrolateral horn of the rat spinal cord decreases from embryonic to adult ages and NTNR-α mRNA is also found in the ventrolateral cell column at embryonic ages. However, in contrast to the results obtained in chicken, NTNR-α transcripts appear to be located in the dorsal horn of the rat spinal cord in the adult (Klein *et al.*, 1997). These differences in the localisation of NTNR-α expression with development may be due to species differences. GDNFR-α and NTNR-α expressing cells in the embryonic chicken and rat spinal cord are likely to be motoneurons, which also express RET mRNA and are known to respond to both GDNF and NTN *in vitro* (Henderson *et al.*, 1994; Treanor *et al.*, 1996; Klein *et al.*, 1997). The loss of motoneurons during the phase of naturally occurring cell death may account for the reduction in the level of expression of these transcripts in the spinal cord with age. In agreement with the results obtained in chicken, GDNFR-α and NTNR-α mRNAs are widely expressed in other regions of the embryonic rodent CNS (Treanor *et al.*, 1996; Klein *et al.*, 1997; Baloh *et al.*, 1997). Thus, GDNFR-α mRNA can be detected by *in situ* hybridization in the developing rat ventral midbrain, pons and medulla oblongata, thalamus, cerebellar primordium, hippocampus, cortex and retina (Treanor *et al.*, 1996), and NTNR-α mRNA expression is localised in the embryonic rodent ventral midbrain (Klein *et al.*, 1997; Baloh *et al.* 1997).

GDNFR-α and NTNR-α transcripts are present in the midbrain where GDNF responsive dopaminergic neurons are located (Treanor *et al.*, 1996; Klein *et al.*, 1997), and in spinal cord where GDNF and neurturin motoneurons are present (Treanor *et al.*, 1996; Klein *et al.*, 1997). It would be interesting to see if the survival of mesencephalic dopaminergic neurons is also promoted by neurturin.

In the peripheral nervous system, GDNFR-α and NTNR-α are also expressed by GDNF and neurturin responsive neurons. Thus, they have been found, using RT/PCR, in purified neurons from embryonic chicken ciliary, nodose, dorsomedial trigeminal and sympathetic ganglia, all of which survive in the presence of either GDNF or neurturin *in vitro* (Trupp *et al.*, 1995; chapters 2 and 3 of this thesis). In agreement with these findings, the expression of both GDNFR-α and NTNR-α mRNAs has been found in embryonic rat dorsal root ganglion, and NTNR-α transcripts are expressed in E15 rat sympathetic ganglia (Treanor *et al.*, 1996; Klein

et al., 1997). Neurons from E15 rat DRG and E21 SCG are supported by both GDNF and neurturin in culture (Kotzbauer *et al.*, 1996).

In non-neuronal tissues of the chicken embryo, GDNFR-α and NTNR-α mRNAs can be detected by Northern blotting at relatively moderate levels in the E10 intestine, kidney, skin, muscle and liver, whereas these transcripts are barely detectable in lung and heart at the same age. GDNFR-α and NTNR-α transcripts are also present in all tissues analysed at E18, although GDNFR-α mRNA was barely detectable by Northern blotting in skin, heart and muscle. In agreement with the results obtained in chicken, GDNFR-α and NTNR-α mRNAs are also widely expressed in non-neuronal tissues of the rat embryo (Treanor *et al.*, 1996; Klein *et al.*, 1997). GDNFR-α can be detected by *in situ* hybridization in several developing tissues, including kidney, intestine, whisker pad and urogenital tract, amongst other regions (Treanor *et al.*, 1996). Moreover, it has been recently shown that NTNR-α transcripts are found in the embryonic gut, bladder, cardiac conduction system and diaphragm (Klein *et al.*, 1997). In rat embryos, GDNFR-α and NTNR-α mRNAs seem to be often co-expressed in the same tissues, occasionally in adjacent structures, suggesting complementary biological roles for GDNF and neurturin (Klein *et al.*, 1997). For example, in the limb GDNFR-α is expressed mainly in muscle cells, whereas NTNR-α is found in the brachial plexus, and in the gut NTNR-α is expressed in the mucosal epithelium and smooth muscle, whereas GDNFR-α is located mainly in the smooth muscle layer (Klein *et al.*, 1997).

RET, the signal tranducing component of the GDNF and neurturin receptor complexes (Trupp *et al.*, 1996; Durbec *et al.*, 1996a; Jing *et al.*, 1996; Treanor *et al.*, 1996; Vega *et al.*, 1996; Worby *et al.*, 1996; Klein *et al.*, 1997; Baloh *et al.*, 1997; this chapter), is co-expressed with GDNFR-α and NTNR-α in most tissues. The analysis of RET expression during embryogenesis has revealed the presence of RET transcripts in several regions of the central nervous system, including pons, medulla, hypothalamus, thalamus, cerebellum, retina, ventral hindbrain and spinal cord (Pachnis *et al.*, 1993; Schuchardt *et al.*, 1995; Tsuzuki *et al.*, 1995; Iwamoto *et al.*, 1993; Trupp *et al.*, 1996). In the peripheral nervous system of chicken and rodent embryos, RET mRNA is expresssed in all cranial ganglia as well in the dorsal root, sympathetic and enteric ganglia (Pachnis *et al.*, 1993; Schuchardt *et al.*,

1995; Tsuzuki *et al.*, 1995). In non-neuronal tissues, RET transcripts have been found in the developing kidney, in particular in the Wolfian duct, ureteric bud epithelium and growing tips of the collecting ducts (Pachnis *et al.*, 1993; Schuchardt *et al.*, 1995; Tsuzuki *et al.*, 1995). Furthermore, RET mRNA is expressed in liver, lung, spleen, thymus, ovary, testis, salivary glands, lymph nodes and chromaffin cells of the adrenal gland (Pachnis *et al.*, 1993; Tsuzuki *et al.*, 1995). In contrast to rodents, RET mRNA is also present in the ventral roots of chicken embryos (Schuchardt *et al.*, 1995). Although embryonic rodent glial cells do not express RET mRNA, they express GDNFR-α and NTNR-α transcripts (Treanor *et al.*, 1996; Baloh *et al.*, 1997). Thus, it would be interesting to elucidate the role that GDNF and neurturin play in these cells.

In agreement with expression studies, mice with targeted disruption of the RET gene display renal agenesis or severe disgenesis, complete neuronal loss of the enteric nervous system in the intestine, whereas the esophagus and stomach are partially affected, and absence of the superior cervical ganglion (Schuchardt *et al.*, 1994; Durbec *et al.*, 1996b). The phenotype of GDNF null mutant mice is very similar to that of RET knockout mice, however, only 35% of neurons are lost in the SCG (Moore *et al.*, 1996). This suggests that other neurotrophic factors, like neurturin, may act physiologically on promoting the survival of this kind of neurons. A detailed analysis of RET deficient mice may clarify whether other neuronal populations of the peripheral nervous system are also affected by this mutation, since 40% and 23% of neurons from the petrosal-nodose and dorsal root ganglia, respectively, are lacking in GDNF knockout mice.

A comprehensive study on the co-expression of GDNFR-α, NTNR-α and RET in several tissues of the chicken during embryogenesis, using Northern blot and *in situ* hybridization, will be necessary to further understand the function of these receptors. Moreover, it would be interesting to analyse whether the level of expression of GDNFR-α, NTNR-α and RET mRNAs in PNS neurons, as determined by quantitative RT/PCR, correlates with the responsiveness of these neurons to GDNF and neurturin throughout development. Preliminary results in this chapter suggest this, since the level of expression of NTNR-α mRNA was higher than GDNFR-α mRNA in ciliary and sympathetic ganglion neurons and these

neurons are more responsive to neurturin than GDNF in culture (chapter 3). In contrast, the level of expression of GDNFR-α mRNA was higher than NTNR-α mRNA in nodose ganglion neurons, which are more responsive to GDNF than neurturin *in vitro* (chapter 3).

In summary, the cloning of both chicken GDNFR-α and NTNR-α has been reported. These homologous proteins are cell-surface receptors that bind preferentially to GDNF and neurturin, respectively, and are bound to the membrane via a glycosyl-phosphatidylinositol linkage. Expression analysis of GDNFR-α and NTNR-α transcripts has revealed that they are widely present in both the nervous system and non-neuronal tissues and are co-expressed by neuronal populations that respond *in vitro* to GDNF and neurturin. The signal transducing component of both GDNF and neurturin receptor complexes is RET, a shared transmembrane tyrosine kinase. This system of sharing receptor components would seem to be an economical way of transducing signals from several growth factors and is also employed by the cytokines (Davis and Yancopoulos, 1993) and members of the TGF-ß protein family (Massagué, 1996a).

XIII. APPENDIX

Table 1. Expression of GDNFR-α and NTNR-α mRNAs by Northern blotting in several chicken tissues at both E10 and E18.

+ : detected
- : not detected
nd. : not determined

	GDNFR-α		NTNR-α	
Total brain	nd.	-	nd.	+
Forebrain	+	+	+	+
Midbrain	+	+	+	+
Pons & Medulla	+	nd.	+	nd.
Cerebellum	+	nd.	+	nd.
Spinal cord	+	+	+	+
Muscle	-	-	+	+
Skin	+	-	+	+
Kidney	+	+	+	+
Total intestine	+	nd.	+	nd.
Small intestine	nd.	+	nd.	+
Large intestine	nd.	+	nd.	+
Heart	-	-	-	-
Liver	+	+	+	+
Lung	-	+	-	+
	E10	**E18**	**E10**	**E18**

Table 2. Expression of GDNFR-α and NTNR-α mRNAs by Northern blotting in chicken midbrain, forebrain, spinal cord and skin between E6 and E16.

+ : detected
- : not detected
nd. : not determined

	GDNFR-α						NTNR-α					
Midbrain	nd.	nd.	+	+	+	nd.	nd.	nd.	+	+	+	nd.
Forebrain	nd.	nd.	+	+	+	nd.	nd.	nd.	+	+	+	nd.
Spinal Cord	nd.	nd.	+	-	-	nd.	nd.	nd.	+	-	-	nd.
Skin	+	+	+	+	+	+	+	+	+	+	+	+
	E6	**E8**	**E10**	**E12**	**E14**	**E16**	**E6**	**E8**	**E10**	**E12**	**E14**	**E16**

Table 3. Expression of GDNFR-α and NTNR-α mRNAs by either in situ hybridization or PCR in several kinds of chicken neurons between E6 and E16.

+ : detected

- : not detected

nd. : not determined

	GDNFR-α			NTNR-α		
Dorsomedial trigeminal g. neurons	nd.	+	nd.	nd.	+	nd.
Sympathetic lumbar chain neurons	+	nd.	nd.	+	nd.	nd.
Nodose ganglion neurons	+	nd.	nd.	+	nd.	nd.
Ciliary ganglion neurons	+	nd.	nd.	+	nd.	nd.
Spinal motoneurons	+	nd.	+	+	nd.	+
Dorsal root ganglion	+	nd.	nd.	+	nd.	nd.
Cerebellar Purkinje cells	nd.	nd.	+	nd.	nd.	+
Cerebellar deep nuclei neurons	nd.	nd.	+	nd.	nd.	+
	E10	**E14**	**E18**	**E10**	**E14**	**E18**

XIV. LIST OF PUBLICATIONS

1. **Buj-Bello, A.**, Pinon, L. G. and Davies, A. M. (1994). The survival of NGF-dependent but not BDNF-dependent cranial sensory neurons is promoted by several different neurotrophins early in their development. Development, 120, 1573-1580.

2. **Buj-Bello, A.**, Buchman, V.L., Horton, A. and Davies, A.M. (1995). GDNF is an age-specific survival factor for sensory and autonomic neurons. Neuron, 15, 821-828.

3. Robinson, M., **Buj-Bello, A.** and Davies, A. M. (1996). Paracrine interactions of BDNF involving NGF-dependent embryonic sensory neurons. Mol. Cell. Neurosci., 7, 143-151.

4. Horton, A. R., Davies, A. M., **Buj-Bello, A.**, Bartlett, P. and Murphy, M. (1996). Leukemia inhibitory factor and ciliary neurotrophic factor in sensory neuron development. Perspectives Dev. Neurobiol., 4, 35-38.

5. Treanor, J. J. S., Goodman, L., de Sauvage, F., Stone, D.M., Poulsen, K.T., Beck, C. D., Gray, C., Armanini, M.P., Pollock, R.A., Hefti, F., Phillips, H.S., Goddard, A., Moore, M.W., **Buj-Bello, A.**, Davies, A.M., Asai, N., Takahashi, M., Vandlen, R., Henderson, C.E. and Rosenthal, A. (1996). Characterization of a multicomponent receptor for GDNF. Nature, 382, 80-83.

6. **Buj-Bello, A.**, Adu, J., Pinon, L.G., Horton, A., Thompson, J., Rosenthal, A., Chinchetru, M., Buchman, V.L., Davies, A.M. (1997). Neurturin responsiveness requires a GPI-linked receptor and the Ret receptor tyrosine kinase. Nature, 387, 721-724.

XV. REFERENCES

Adler, R., Landa, K. B., Manthorpe, M. and Varon, S. (1979) Cholinergic neuronotrophic factors: intraocular distribution of soluble trophic factor activity for ciliary neurons. Science, 204, 1434-1436.

Alderson, R. F., Alterman, A. L., Barde, Y. A. and Lindsay, R. M. (1990) Brain-derived neurotrophic factor increases survival and differentiated functions of rat septal cholinergic neurons in culture. Neuron, 5, 297-306.

Allsopp, T. E., Robinson, M., Wyatt, S. and Davies, A. M. (1993) Ectopic trkA expression mediates a NGF survival response in NGF-independent sensory neurons but not in parasympathetic neurons. J. Cell Biol., 123, 1555-1566.

Angeletti, R. H. and Bradshaw, R. A. (1971) Nerve growth factor from mouse submaxillary gland: amino acid sequence. Proc. Natl. Acad. Sci., 68,

Arakawa, Y., Sendtner, M. and Thoenen, H. (1990) Survival effect of ciliary neurotrophic factor (CNTF) on chick embryonic motoneurons in culture: comparison with other neurotrophic factors and cytokines. J. Neurosci., 10, 3507-3515.

Arenas, E. and Persson, H. (1994) Neurotrophin-3 prevents the death of adult central noradrenergic neurons in vivo. Nature, 367, 368-371.

Arenas, E., Trupp, M., Akerud, P. and Ibanez, C. F. (1995) GDNF prevents degeneration and promotes the phenotype of brain noradrenergic neurons in vivo. Neuron, 15, 1465-1473.

Arighi, E., Alberti, L., Torriti, F., Ghizzoni, F., Rizzetti, M. G., Pelicci, G., Pasini, B., Bongarzone, I., Piutti, C., Pierotti, M. A. and Borello, M. G. (1997) Identification of Shc docking site on Ret tyrosine kinase. Oncogene, 14, 773-782.

Attié, T., Till, M., Pelet, A., Amiel, J., Edery, P., Boutrand, L., Munnich, A. and Lyonnet, S. (1995) Mutation of the endothelin receptor-B gene in Waardenburg-Hirschsprung disease. Hum. Mol. Genet., 4, 2407-2409.

Attisano, L., Carcamo, J., Ventura, F., Weis, F. M. B., Massagué, J. and Wrana, J. L. (1993) Identification of human activin and TGFß type I receptors that form heteromeric kinase complexes with type II receptors. Cell, 75, 671-680.

Attisano, L., Wrana, J. L., Cheifetz, S. and Massagué, J. (1992) Novel activin receptors: distinct genes and alternative mRNA splicing generate a repertoire of serine/threonine kinase receptors. Cell, 68, 97-108.

Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., J.G., S., Smith, J. A. and Struhl, K. (1995) Current Protocols in Molecular Biology. In Massachusetts General Hospital. Harvard Medical School. John Wiley &Sons Inc.

Averbuch, H. L., Pruginin, M., Kahane, N., Tsoulfas, P., Parada, L., Rosenthal, A. and Kalcheim, C. (1994) Neurotrophin 3 stimulates the differentiation of motoneurons from avian neural tube progenitor cells. Proc. Natl. Acad. Sci. USA, 91, 3247-3251.

Bakhshi, A., Jensen, J. P., Goldman, P., Wright, J. J., McBride, O. W., Epstein, A. L. and Korsmeyer, S. J. (1985) Cloning the chromosomal breakpoint of t(14;18) human lymphomas: clustering around JH on chromosome 14 and near a transcriptional unit on 18. Cell, 41, 889-906.

Baloh, R.H., Tansey, M.G., Golden, J.P., Creedon, D.J., Heuckeroth, R.O., Keck, C.L., Zimonjic, D.B., Popescu, N.C., Johnson, E.M.Jr., Milbrandt, J. (1997) TrnR2, a novel receptor that mediates neuturin and GDNF signalling through RET. Neuron, 18, 793-802.

Banner, L. R. and Patterson, P. H. (1994) Major changes in the expression of the mRNAs for cholinergic differentiation factor/leukemia inhibitory factor and its receptor after injury to adult peripheral nerves and ganglia. Proc. Natl. Acad. Sci. USA, 91, 7109-7113.

Barbin, G., Manthorpe, M. and Varon, S. (1984) Purification of the chick eye ciliary neuronotrophic factor. J. Neurochem., 43, 1468-1478.

Barde, Y. A. (1989) Trophic factors and neuronal survival. Neuron, 2, 1525-1534.

Barde, Y. A. (1994) Neurotrophic factors: an evolutionary perspective. J. Neurobiol., 25, 1329-1333.

Barde, Y. A., Davies, A. M., Johnson, J. E., Lindsay, R. M. and Thoenen, H. (1987) Brain derived neurotrophic factor. Prog. Brain Res., 71, 185-189.

Barde, Y. A., Edgar, D. and Thoenen, H. (1982) Purification of a new neurotrophic factor from mammalian brain. EMBO J., 1, 549-553.

Barker, P. A., Lomen-Hoerth, C., Gensch, E. M., Meakin, S. O., Glass, D. J. and Shooter, E. M. (1993) Tissue-specific alternative splicing generates two isoforms of the trkA receptor. J. Biol. Chem., 268, 15150-15157.

Barres, B. A., Raff, M. C., Gaese, F., Bartke, I., Dechant, G. and Barde, Y. A. (1994) A crucial role for neurotrophin-3 in oligodendrocyte development. Nature, 367, 371-375.

Baynash, A. G., Hosoda, K., Giaid, A., Richardson, J. A., Emoto, N., Hammer, R. E. and Yanagisawa, M. (1994) Interaction of endothelin-3 with endothelin-B receptor is essential for development of epidermal melanocytes and enteric neurons. Cell, 79, 1277-1285.

Bazan, J. F. (1991) Neuropoietic cytokines in the hematopoietic fold. Neuron, 7, 197-208.

Beck, K. D., Valverde, J., Alexi, T., Poulsen, K., Moffat, B., Vandlen, R. A., Rosenthal, A. and Hefti, F. (1995) Mesencephalic dopaminergic neurons protected by GDNF from axotomy-induced degeneration in the adult brain. Nature, 373, 339-341.

Berger, E. A. and Shooter, E. M. (1977) Evidence for pro-ß-nerve growth factor, a biosynthetic precursor to ß-nerve growth factor. Proc. Natl. Acad. Sci. USA, 74, 3647-3651.

Berkemeier, L. R., Winslow, J. W., Kaplan, D. R., Nikolics, K., Goeddel, D. V. and Rosenthal, A. (1991) Neurotrophin-5: a novel neurotrophic factor that activates trk and trkB. Neuron, 7, 857-866.

Birboim, H. C. (1983) A rapid alkaline extraction method for the isolation of plasmid DNA. Methods Enzymol., 100, 243-255.

Birboim, H. C. and Doly, J. (1979) A rapid alkaline extraction method for screening recombinant plasmid DNA. Nucl. Acids Res., 7, 1513-1523.

Birren, S. J., Lo, L. and Anderson, D. J. (1993) Sympathetic neuroblasts undergo a developmental switch in trophic dependence. Development, 119, 597-610.

Bongarzone, I., Butti, M. G., Coronelli, S., Borrello, M. G., M., S., Mondellini, P., Pilotti, S., Fusco, A., Della Porta, G. and Pierotti, M. A. (1994) Frequent activation of ret proto-oncogene by fusion with a new activating gene in papillary thyroid carcinomas. Cancer Res., 54, 2979-2985.

Bongarzone, I., Monzini, N., Borello, M. G., Careano, C., Ferraresi, G., Arighi, E., Mondellini, P., Della-Porta, G. and Pierotti, M. A. (1993) Molecular characterization of a thyroid tumor-specific transforming sequence formed by the fusion of ret tyrosine kinase and the regulatory subunit RI alpha of cyclic AMP-dependent protein kinase A. Mol. Cell Biol., 13, 358-366.

Bonni, A., Frank, D. A., Schindler, C. and Greenberg, M. E. (1993) Characterization of a pathway for ciliary neurotrophic factor signaling to the nucleus. Science, 262, 1575-1579.

Borello, M. G., Pelicci, G., Arighi, E., Defilippis, L., Greco, A., Bongarzone, I., Rizzetti, M. G., Pelicci, P. G. and Pierotti, M. A. (1994) The oncogenic versions of the Ret and Trk tyrosine kinases bind Shc and Grb2 adapter proteins. Oncogene, 9, 1661-1668.

Bowenkamp, K. E., David, D., Lapchak, P. L., Henry, M. A., Granholm, A. C., Hoffer, B. J. and Mahalik, T. J. (1996) 6-hydroxydopamine induces the loss of the dopaminergic phenotype in substantia nigra neurons of the rat. Exp. Brain Res., 111, 1-7.

Bowenkamp, K. E., Hoffman, A. F., Gerhardt, G. A., Henry, M. A., Biddle, P. T., Hoffer, B. J. and Granholm, A. C. E. (1995) Glial cell line-derived neurotrophic factor supports survival of injured midbrain dopaminergic neurons. J. Comp. Neurol., 355, 479-489.

Bronner-Fraser, M. (1988) Guidance of neural crest migration during the early development of the peripheral nervous system. Oxford University Press, Oxford.

Bruce, A. G., Linsley, P. S. and Rose, T. M. (1992) Oncostatin M. Prog. Growth Factor Res., 4, 157-170.

Brummel, T. J., Twombly, V., Marqués, G., Wrana, J. L., Newfeld, S. J., Attisano, L., Massagué, J., O'Connor, M. B. and Gelbart, W. M. (1994) Characterization and relationship of dpp receptors encoded by the saxophone and thick veins genes in Drosophila. Cell, 78, 251-261.

Buchman, V. L. and Davies, A. M. (1993) Different neurotrophins are expressed and act in a developmental sequence to promote the survival of embryonic sensory neurons. Development, 118, 989-1001.

Buchman, V. L., Sporn, M. and Davies, A. M. (1994) Role of transforming growth factor-beta isoforms in regulating the expression of nerve growth factor and neurotrophin-3 mRNA levels in embryonic cutaneous cells at different stages of development. Development, 120, 1621-1629.

Buj-Bello, A., Pinon, L. G. and Davies, A. M. (1994) The survival of NGF-dependent but not BDNF-dependent cranial sensory neurons is promoted by several different neurotrophins early in their development. Development, 120, 1573-1580.

Buj-Bello, A., Buchman, V.L., Horton, A. and Davies, A.M. (1995) GDNF is an age-specific survival factor for sensory and autonomic neurons. Neuron, 15, 821-828.

Buj-Bello, A., Adu, J., Pinon, L.G., Horton, A., Thompson, J., Rosenthal, A., Chinchetru, M., Buchman, V.L., Davies, A.M. (1997) Neurturin responsiveness requires a GPI-linked receptor and the Ret receptor tyrosine kinase. Nature, 387, 721-724.

Carlomagno, F., De Vita, G., Berlingieri, M. T., de Franciscis, V., Melillo, R. M., Colantuoni, V., Kraus, M. H., Di Fiore, P. P., Fusco, A. and Santoro, M. (1996) Molecular heterogeneity of RET loss of function in Hirschsprung's disease. EMBO J., 15, 2717-2725.

Carlson, K. M., Dou, S., Chi, D., Scavarda, N., Toshima, K., Jackson, C. E., Wells, J. S. A., Goodfellow, P. J. and Donis-Keller, H. (1994) Single missense mutation in the tyrosine kinase catalytic domain of the RET protooncogene is associated with multiple endocrine neoplasia type 2B. Proc. Natl. Acad. Sci. USA, 91, 1579-1583.

Carter, B. D. and Lewin, G. R. (1997) Neurotrophins live or let die: does p75NTR decide? Neuron, 18, 187-190.

Cassaccia-Bonnefil, P., Carter, B. D., Dobrowsky, R. T. and Chao, M. V. (1996) Death of oligodendrocytes mediated by the interaction of nerve growth factor with its receptor p75. Nature, 383, 716-719.

Chalazonitis, A., Kalberg, J., Twardzik, D. R., Morrison, R. S. and Kessler, J. A. (1992) Transforming growth factor ß has neurotrophic actions on sensory neurons in vitro and is synergistic with nerve growth factor. Dev. Biol., 152, 121-132.

Chao, M. V., Bothwell, M. A., Ross, A. H., Koprowski, H., Lanahan, A. A., Buck, C. R. and Sehgal, A. (1986) Gene transfer and molecular cloning of the human NGF receptor. Science, 232, 518-521.

Chao, M. V. and Hempstead, B. L. (1995) p75 and TrK: a two-receptor system. Trends Neurosci., 18, 321-326.

Chizgwin, J. M., Przybyla, A. E., McDonald, R. J. and Rutter, W. J. (1979) Isolation of biologically active ribonucleic acid from sources enriched in ribonucleases. Biochemistry, 18, 5294-5299.

Chomczynski, P. and Sacchi, N. (1987) Single step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem., 162, 156-159.

Chun, L. L. and Patterson, P. H. (1977) Role of nerve growth factor in the development of rat sympathetic neurones in vitro. II. Developmental studies. J. Cell Biol., 75, 705-711.

Clatterbuck, R. E., Price, D. L. and Koliatsos, V. E. (1993) Ciliary neurotrophic factor prevents retrograde neuronal death in the adult central nervous system. Proc. Natl. Acad. Sci. USA, 90, 2222-2226.

Cleary, M. L., Smith, S. D. and Sklar, J. (1986) Cloning and structural analysis of cDNAs for bcl-2 and a hybrid bcl-2/immunoglobulin transcript resulting from the t(14;18) translocation. Cell, 47, 19-28.

Cohen, S. (1960) Purification of a nerve-growth promoting protein from the mouse salivary gland and its neuro-cytotoxic anstiserum. Proc. Natl. Acad. Sci. USA, 46, 302-311.

Conover, J. C., Erickson, J. T., Katz, D. M., Bianchi, L. M., Poueymirou, W. T., McClain, J., Pan, L., Helgren, M., Ip, N. Y., Boland, P., Friedman, B., Wiegand, S., Vejsada, R., Kato, A. C., DeClara, T. M. and Yancopoulos, G. D. (1995) Neuronal deficits, not involving motor neurons, in mice lacking BDNF and NT4. Nature, 375, 235-238.

Cristina, N., Chatellard-Causse, C., Manier, M. and Feuerstein, C. (1995) GDNF: existence of a second transcript in the brain. Mol. Brain Res., 32, 354-357.

Crowley, C., Spencer, S. D., Nishimura, M. C., Chen, K. S., Pitts-Meek, S., Armanini, M. P., Ling, L. H., McMahon, S. B., Shelton, D. L., Levinson, A. D. and Phillips, H. S. (1994) Mice lacking nerve growth factor display perinatal loss of sensory and sympathetic neurons yet develop basal forebrain cholinergic neurons. Cell, 76, 1001-1011.

Curtis, R., Adryan, K. M., Zhu, Y., Harkness, P. J., Lindsay, R. M. and DiStefano, P. S. (1993) Retrograde axonal transport of ciliary neurotrophic factor is increased by peripheral nerve injury. Nature, 365, 253-255.

Curtis, R., Scherer, S. S., Somogyi, R., Adryan, K. M., Ip, N. Y., Zhu, Y., Lindsay, R. M. and DiStefano, P. S. (1994) Retrograde axonal transport of LIF is increased by peripheral nerve injury: correlation with increased LIF expression in distal nerve. Neuron, 12, 191-204.

D'Amico, M. A. and Noden, D. M. (1983) Contributions of placodal and neural crest cells to avian cranial peripheral ganglia. Am. J. Anat., 166, 445-468.

Daopin, S., Piez, K. A., Ogawa, Y. and Davies, D. R. (1992) Crystal structure of transforming growth factor-ß2: an unusual fold for the superfamily. Science, 257, 369-373.

Davies, A. M. (1986b) The survival and growth of embryonic proprioceptive neurons is promoted by a factor present in skeletal muscle. Dev. Biol., 115, 56-67.

Davies, A. M. (1987b) Molecular and cellular aspects of patterning sensory neurone connections in the vertebrate nervous system. Development, 101, 185-208.

Davies, A. M. (1988a) In Rush, R. (ed.), Nerve Growth Factors. pp. 95-109.

Davies, A. M. (1988b) Role of neurotrophic factors in development. Trends Genet., 4, 139-143.

Davies, A. M. (1994a) Role of neurotrophins in the developing nervous system. J. Neurobiol., 25, 1334-1348.

Davies, A. M. (1994b) The role of neurotrophins during successive stages of sensory neuron development. Prog. Growth Factor Res., 5, 263-289.

Davies, A. M. (1994c) Tracking neurotrophin function. Nature, 368, 193-194.

Davies, A. M. (1995) The Bcl-2 family of proteins and the regulation of neuronal survival. Trends Neurosci., 18, 355-358.

Davies, A. M. (1996a) Paracrine and autocrine actions of neurotrophic factors. Neurochem. Res., 21, 749-753.

Davies, A. M. (1996b) The yin and yang of nerve growth factor. Curr. Biol., 7,

Davies, A. M., Bandtlow, C., Heumann, R., Korsching, S., Rohrer, H. and Thoenen, H. (1987) Timing and site of nerve growth factor synthesis in developing skin in relation to innervation and expression of the receptor. Nature, 326, 353-358.

Davies, A. M., Horton, A., Burton, L. E., Schmelzer, C., Vandlen, R. and Rosenthal, A. (1993a) Neurotrophin-4/5 is a mammalian-specific survival factor for distinct populations of sensory neurons. J. Neurosci., 13, 4961-4967.

Davies, A. M. and Lindsay, R. M. (1985) The cranial sensory ganglia in culture: Differences in the response of placode-derived and neural crest-derived neurons to nerve growth factor. Dev. Biol., 111, 62-72.

Davies, A. M., Thoenen, H. and Barde, Y. A. (1986a) The response of chick sensory neurons to brain-derived neurotrophic factor. J. Neurosci., 6, 1897-1904.

Davis, S., Aldrich, T. H., Ip, N. Y., Stahl, N., Scherer, S., Farruggella, T., DiStefano, P. S., Curtis, R., Panayotatos, N., Gascan, H., Chelalier, S. and Yancopoulos, G. D. (1993b) Released form of CNTF receptor alpha component as a soluble mediator of CNTF responses. Science, 259, 1736-1739.

Davis, S., Aldrich, T. H., Stahl, N., Pan, L., Taga, T., Kishimoto, T., Ip, N. Y. and Yancopoulos, G. D. (1993a) LIFRß and gp130 as heterodimerizing signal transducers of the tripartite CNTF receptor. Science, 260, 1805-1808.

Davis, S., Aldrich, T. H., Valenzuela, D. M., Wong, V., Furth, M. E., Squinto, S. P. and Yancopoulos, G. D. (1991) The receptor for ciliary neurotrophic factor. Science, 253, 59-63.

Davis, S. and Yancopoulos, G. D. (1993) The molecular biology of the CNTF receptor. Curr. Opin. Neurobiol., 3, 20-24.

DeChiara, T. M., Vejsada, R., Poueymirou, W. T., Acheson, A., Suri, C., Conover, J. C., Friedman, B., McClain, J., Pan, L., Stahl, N., Ip, N. Y., Kato, A. and Yancopoulos, G. D. (1995) Mice lacking the CNTF receptor, unlike mice lacking CNTF, exhibit profound
motor neuron deficits at birth. Cell, 83, 313-322.

DiCicco-Bloom, E., Friedman, W. J. and Black, I. B. (1993) NT-3 stimulates sympathetic neuroblast proliferation by promoting precursor cell survival. Neuron, 11, 1101-1111.

Dobrea, G. M., Unnerstall, J. R. and Rao, M. S. (1992) The expression of CNTF message and immunoreactivity in the central and peripheral nervous system of the rat. Devel. Brain Res., 66, 209-219.

Durbec, P., Marcos-Gutierrez, C. V., Kilkenny, C., Grigoriou, M., Wartiowaara, K., Suvanto, P., Smith, D., Ponder, B., Costantini, F., Saarma, M., Sariola, H. and Pachnis, V. (1996a) GDNF signalling through the Ret receptor tyrosine kinase. Nature, 381, 789-793.

Durbec, P.L., Larsson-Blomberg, L.B., Schuchardt, A., Costantini, F. (1996b) Common origin and developmental dependence on c-ret of subsets of enteric and sympathetic neuroblasts. Development, 122, 349-358.

Ebendal, T., Larhammar, D. and Persson, H. (1986) Structure and expression of the chicken ß nerve growth factor gene. Embo J., 5, 1483-1487.

Ebner, R., Chen, R. H., Shum, L., Lawler, S., Zioncheck, T. F., Lee, A., Lopez, A. R. and Derynck, R. (1993) Cloning of a type I TGF-ß receptor and its effects on TGF-ß binding to the type II receptor. Science, 260, 1344-1348.

Eckenstein, F. P. (1994) Fibroblast growth factors in the nervous system. J. Neurobiol., 25, 1467-1480.

Eckenstein, F. P., Esch, F., Holbert, T., Blacher, R. W. and Nishi, R. (1990) Purification and characterization of a trophic factor for embryonic peripheral neurons: comparison with fibroblast growth factors. Neuron, 4, 623-631.

Edery, P., Lyonnet, S., Mulligan, L. M., Pelet, A., Dow, E., Abel, L., Holder, S., Nihoul-Fékété, C., Ponder, B. A. J. and Munnich, A. (1994) Mutations of the RET proto-oncogene in Hirschsprung's disease. Nature, 367, 378-380.

Edwards, R. H., Selby, M. J., Garcia, P. D. and Rutter, W. J. (1988) Processing of the native nerve growth factor precursor to form biologically active nerve growth factor. J. Biol. Chem., 263, 6810-6815.

Epstein, M. L., Mikawa, T., Brown, A. M. C. and McFarlin, D. R. (1994) Mapping the origin of the avian enteric nervous system with a retroviral marker. Dev. Dyn., 201, 236-244.

Ernfors, P., Ibanez, C. F., Ebendal, T., Olson, L. and Persson, H. (1990) Molecular cloning and neurotrophic activities of a protein with structural similarities to nerve growth factor: developmental and topographical expression in the brain. Proc. Natl. Acad. Sci. USA, 87, 5454-5458.

Ernfors, P., Lee, K. F. and Jaenisch, R. (1994a) Mice lacking brain-derived neurotrophic factor develop with sensory deficits. Nature, 368, 147-150.

Ernfors, P., Lee, K. F., Kucera, J. and Jaenisch, R. (1994b) Lack of neurotrophin-3 leads to deficiencies in the peripheral nervous system and loss of limb proprioceptive afferents. Cell, 77, 503-512.

Ernsberger, U., Edgar, D. and Rohrer, H. (1989b) The survival of early chick sympathetic neurons in vitro is dependent on a suitable substrate but independent of NGF. Devel. Biol., 135, 250-262.

Ernsberger, U., Sendtner, M. and Rohrer, H. (1989a) Proliferation and differentiation of embryonic chick sympathetic neurons: effects of ciliary neurotrophic factor. Neuron, 2, 1275-1284.

Fan, G. and Katz, D. M. (1993) Non-neuronal cells inhibit catecholaminergic differentiation of primary sensory neurons: role of leukemia inhibitory factor. Development, 118, 83-93.

Fan, M. J. and Patterson, P. H. (1994) Neuropoietic cytokines and activin A differentially regulate the phenotype of cultured sympathetic neurons. Proc. Natl. Acad. Sci. USA, 91, 43-47.

Farinas, I., Jones, K. R., Backus, C., Wang, X. Y. and Reichardt, L. F. (1994) Severe sensory and sympathetic deficits in mice lacking neurotrophin-3. Nature, 369, 658-661.

Frade, J. M., Rodriguez-Tebar, A. and Barde, Y. A. (1996) Induction of cell death by endogenous nerve growth factor through its p75 receptor. Nature, 383, 166-168.

Franzén, P., Dijke, P. T., Ichijo, H., Yamashita, H., Schulz, P., Heldin, C. H. and Miyazono, K. (1993) Cloning of a TGFß type I receptor that forms a heteromeric complex with the TGFß type II receptor. Cell, 75, 681-692.

Fraser, A. and Evan, G. (1996) A license to kill. Cell, 85, 781-784.

Friedman, W. J., Ibanez, C. F., Hallbook, F., Persson, H., Cain, L. D., Dreyfus, C. F. and Black, I. B. (1993) Differential actions of neurotrophins in the locus coeruleus and basal forebrain. Exp. Neurol., 119, 72-78.

Fukada, K. (1985) Purification and partial characterization of a cholinergic neuronal differentiation factor. Proc. Natl. Acad. Sci. USA, 82, 8795-8799.

Gajewski, T. and Thompson, C. B. (1996) Apoptosis meets signal transduction: elimination of a BAD influence. Cell, 87,

Gash, D. M., Zhang, Z., Ovadia, A., Cass, W. A., Yi, A., Simmerman, L., Russell, D., Martin, D., Lapchak, P. A., Collins, F., Hoffer, B. J. and Gerhardt, G. A. (1996) Functional recovery in parkinsonian monkeys treated with GDNF. Nature, 380, 252-255.

Gearing, D. P., Comeau, M. R., Friend, D. J., Gimpel, S. D., Thut, C. J., McGourty, J., Brasher, K. K., King, J. A., Gillis, S., Mosley, B., Ziegler, S. F. and Cosman, D. (1992) The IL-6 signal transducer, gp130: an Oncostatin M receptor and affinity converter for LIF receptor. Science, 255, 1434-1437.

Gearing, D. P., Gough, N. M., King, J. A., Hilton, D. J., Nicola, N. A., Simpson, R. J., Nice, E. C., Kelso, A. and Metcalf, D. (1987) Molecular cloning and expression of cDNA encoding a murine myeloid leukaemia inhibitory factor (LIF). EMBO J., 6, 3995-4002.

Gearing, D. P., Thut, C. J., Vandenbos, T., Gimpel, S. D., Delaney, P. B., King, J., Price, V., Cosman, D. and Beckmann, M. P. (1991) Leukemia inhibitory factor receptor is structurally related to the IL-6 signal transducer, gp130. EMBO J., 10, 2839-2848.

Gerber, L. D., Kodukula, K. and Udenfriend, S. (1992) Phosphatidylinositol glycan (PI-G) anchored membrane proteins. Amino acid requirements adjacent to the site of cleavage and PI-G attachment in the COOH-terminal signal peptide. J. Biol. Chem., 267, 12168-12173.

Golstein, P. (1997) Controlling cell death. Science, 275, 1081-1082.

Gotz, R., Koster, R., Winkler, C., Raulf, F., Lottspeich, F., Schartl, M. and Thoenen, H. (1994) Neurotrophin-6 is a new member of the nerve growth factor family. Nature, 372, 266-269.

Gouin, A., Bloch-Gallego, E., Tanaka, H., Rosenthal, A. and Henderson, C. E. (1996) Transforming growth factor-beta 3, glial cell line-derived neurotrophic factor, and fibroblast growth factor-2, act in different manners to promote motoneuron survival in vitro. J. Neurosci. Res., 43, 454-464.

Graff, J. M., Bansal, A. and Melton, D. A. (1996) Xenopus Mad proteins transduce distinct subsets of signals for the TGFß superfamily. Cell, 85, 479-487.

Grieco, M., Santoro, M., Berlingieri, M. T., Melillo, R. M., Donghi, R., Bongarzone, I., Pierotti, M. A., Della-Porta, G., Fusco, A. and Vecchio, G. (1990) PTC is a novel rearranged form of the ret proto-oncogene and is frequently detected in vivo in human thyroid papillary carcinomas. Cell, 60, 557-563.

Ha, D. H., Robertson, R. T., Ribak, C. E. and Weiss, J. H. (1996) Cultured basal forebrain cholinergic neurons in contact with cortical cells display synapses, enhanced morphological features, and decreased dependence on nerve growth factor. J. Comp. Neurol., 373, 451-465.

Hagg, T. and Varon, S. (1993) Ciliary neurotrophic factor prevents degeneration of adult rat substantia nigra dopaminergic neurons in vivo. Proc. Natl. Acad. Sci. USA, 90, 6315-6319.

Hall, A. and Rao, M. (1992) Cytokines and neurokines: related ligands and related receptors. Trends Neurosci., 15, 35-37.

Hallböök, F., Ibanez, C. F. and Persson, H. (1991) Evolutionary studies of the nerve growth factor family reveal a novel member abundantly expressed in Xenopus ovary. Neuron, 6, 845-858.

Hama, T., Miyamoto, M., Tsukui, H., Nishio, C. and Hatanaka, H. (1989) Interleukin-6 as a neurotrophic factor for promoting the survival of cultured basal forebrain cholinergic neurons from postnatal rats. Neurosci. Lett., 104, 340-344.

Hamburger, V., Brunso-Bechtold, J. K. and Yip, J. W. (1981) Neuronal death in the spinal ganglia of the chick embryo and its reduction by nerve growth factor. J. Neurosci., 1, 60-71.

Hamburger, V. and Hamilton, H. L. (1951) A series of normal stages in the developmentof the chick embryo. J. Morphol., 88, 49-92.

Harper, S. and Davies, A. (1990) NGF mRNA expression in developing cutaneous epithelium related to innervation density. Development, 110, 515-519.

Hartikka, J. and Hefti, F. (1988) Comparison of nerve growth factor's effects on development of septum, striatum, and nucleus basalis cholinergic neurons in vitro. J. Neurosci. Res., 21, 352-364.

Helgren, M. E., Squinto, S. P., Davis, H. L., Parry, D. J., Boulton, T. G., Heck, C. S., Zhu, Y., Yancopoulos, G. D., Lindsay, R. M. and DiStefano, P. S. (1994) Trophic effect of ciliary neurotrophic factor on denervated skeletal muscle. Cell, 76, 493-504.

Heller, S., Huber, J., Finn, T. P., Nishi, R. and Rohrer, H. (1993) GPA and CNTF produce similar effects in sympathetic neurones but differ in receptor binding. Neuroreport, 5, 357-360.

Henderson, C. E., Camu, W., Mettling, C., Gouin, A., Poulsen, K., Karlaloo, M., Rullamas, J., Evans, T., McMahon, S. B., Armanini, M. P., Berkemeier, L., Phillips, H. S. and Rosenthal, A. (1993) Neurotrophins promote motor neuron survival and are present in embryonic limb bud. Nature, 363, 266-270.

Henderson, C. E., Phillips, H. S., Pollock, R. A., Davies, A. M., Lemeulle, C., Armanini, M., Simpson, L. C., Moffet, B., Vandlen, R. A., Koliatsos, V. E. and Rosenthal, A. (1994) GDNF: A potent survival factor for motoneurons present in peripheral nerve and muscle. Science, 266, 1062-1064.

Hirano, T. (1991) The cytokine handbook. In Thomson, A. (ed.). Academic press, London, pp. 169-190.

Hofer, M., Pagliusi, S. R., Hohn, A., Leibrock, J. and Barde, Y. A. (1990) Regional distribution of brain-derived neurotrophic factor mRNA in the adult mouse brain. Embo J, 9, 2459-2464.

Hofer, M. M. and Barde, Y. A. (1988) Brain-derived neurotrophic factor prevents neuronal death in vivo. Nature, 331, 261-262.

Hoffer, B. J., Hoffman, A., Bowenkamp, K., Huettl, P., Hudson, J., Martin, D., Lin, L. F. H. and G.A., G. (1994) Glial cell line-derived neurotrophic factor reverses toxin-induced injury to midbrain dopaminergic neurons in vivo. Neurosci. lett., 182, 107-111.

Hoffmann, F. M. (1991) Transforming growth factor-ß-related genes in Drosophila and vertebrate development. Curr. Op. Cell Biol., 3, 947-952.

Hofstra, R. M. W., Landsvater, R. M., Ceccherini, I., Stulp, R. P., Stelwagen, T., Luo, Y., Pasini, B., Höppener, J. W. M., van Amstel, H. K. P., Romeo, G., Lips, C. X. and Buys, C. H. C. M. (1994) A mutation in the RET proto-oncogene associated with multiple endocrine neoplasia type 2B and sporadic medullary thyroid carcinoma. Nature, 367, 375-376.

Hogan, B. L. M. (1996) Bone morphogenetic proteins: multifunctional regulators of vertebrate development. Genes & Dev., 10, 1580-1594.

Hohn, A., Leibrock, J., Bailey, K. and Barde, Y. A. (1990) Identification and characterization of a novel member of the nerve growth factor/brain-derived neurotrophic factor family. Nature, 344, 339-341.

Holmes, D. S. and Quigley. (1981) A rapid boiling method for the preparation of bacterial plasmids. Anal. Biochem., 14, 193-197.

Horton, A. R., Bartlett, P. F., Pennica, D. and Davies, A. M. (1997) Neurotrophic cytokines promote the survival of cranial sensory neurons at different stages of their development (submitted).

Horton, A. R., Davies, A. M., Buj-Bello, A., Bartlett, P. and Murphy, M. (1996) Leukemia inhibitory factor and ciliary neurotrophic factor in sensory neuron development. Perspectives Dev. Neurobiol., 4, 35-38.

Houenou, L. J., Oppenheim, R. W., Li, L., Lo, A. C. and Prevette, D. (1996) Regulation of spinal motoneuron survival by GDNF during development and following injury. Cell Tissue Res., 286, 219-221.

Hudson, J., Granholm, A. C., Gerhardt, G. A., Henry, M. A., Hoffman, A., Biddle, P., Leela, N. S., Mackerlova, L., Lile, J. D., Collins, F. and Hoffer, B. J. (1995) Glial cell line-derived neurotrophic factor augments midbrain dopaminergic circuits in vivo. Brain Res. Bull., 36, 425-432.

Hughes, S. M., Lillien, L. E., Raff, M. C., Rohrer, H. and Sendtner, M. (1988) Ciliary neurotrophic factor induces type-2 astrocyte differentiation in culture. Nature, 335, 70-73.

Hyman, C., Hofer, M., Barde, Y. A., Juhasz, M., Yancopoulos, G. D., Squinto, S. P. and Lindsay, R. M. (1991) BDNF is a neurotrophic factor for dopaminergic neurons of the substantia nigra. Nature, 350, 230-232.

Ibanez, C. F., Ernfors, P., Timmusk, T., Ip, N. Y., Arenas, E., Yancopoulos, G. D. and Persson, H. (1993) Neurotrophin-4 is a target-derived neurotrophic factor for neurons of the trigeminal ganglion. Development, 117, 1345-1353.

Ip, N. Y., Ibanez, C. F., Nye, S. H., McClain, J., Jones, P. F., Gies, D. R., Belluscio, L., LeBeau, M., Espinosa, R., Squinto, S. P., Persson, H. and Yancopoulas, G. D. (1992a) Mammalian neurotrophin-4: structure, chromosomal localization, tissue distribution, and receptor specificity. Proc. Natl. Acad. Sci. USA, 89, 3060-3064.

Ip, N. Y., Li, Y. P., Van de Stadt, I., Panayotatos, N., Alderson, R. F. and Lindsay, R. M. (1991) Ciliary neurotrophic factor enhances neuronal survival in embryonic rat hippocampal cultures. J. Neurosci., 11, 3124-3134.

Ip, N. Y., McClain, J., Barrezueta, N. X., Aldrich, T. H., Pan, L., Li, Y., Wiegand, S., Friedman, B., Davis, S. and Yancopoulos, G. D. (1993b) The a-component of the CNTF receptor is required for signaling and defines potential CNTF targets in the adult and during development. Neuron, 10, 89-102.

Ip, N. Y., Nye, S. H., Boulton, T. G., Davis, S., Taga, T., Li, Y., Birren, S. J., Yasukawa, K., Kishimoto, T., Anderson, D. J., Stahl, N. and Yancopoulos, G. D. (1992b) CNTF and LIF act on neuronal cells via shared signaling pathways that involve the IL-6 signal transducing receptor component gp130. Cell, 69, 1121-1132.

Ip, N. Y., Wiegand, S. J., Morse, J. and Rudge, J. S. (1993a) Injury-induced regulation of ciliary neurotrophic factor mRNA in the adult rat brain. Eur. J. Neurosci., 5, 25-33.

Ivanchuk, S. M., Myers, S. M., Eng, C. and Mulligan, L. M. (1996) De novo mutation of GDNF, ligand for the RET/GDNFR-alpha receptor complex, in Hirschsprung disease. Hum. Mol. Genet., 5, 2023-2026.

Iwamoto, T., Taniguchi, M., Asai, N., Ohkusu, K., Nakashima, I. and Takahashi, M. (1993) cDNA cloning of mouse ret proto-oncogene and its sequence similarity to the cadherin superfamily. Oncogene, 8, 1087-1091.

Jing, S., Wen, D., Yu, Y., Holst, P. L., Luo, Y., Fang, M., Tamir, R., Antonio, L., Hu, Z., Cupples, R., Louis, J. C., Hu, S., Altrock, B. W. and Fox, G. M. (1996) GDNF-induced activation of the Ret protein tyrosine kinase is mediated by GDNFR-a, a novel receptor for GDNF. Cell, 85, 1113-1124.

Johansson, M., Friedemann, M., Hoffer, B. and Strömberg, I. (1995) Effects of glial cell line-derived neurotrophic factor on developing and mature ventral mesencephalic grafts in oculo. Exp. Neurol., 134, 25-34.

Jones, K. R., Farinas, I., Backus, C. and Reichardt, L. F. (1994) Targeted disruption of the BDNF gene perturbs brain and sensory neuron development but not motor neuron development. Cell, 76, 989-999.

Jones, K. R. and Reichardt, L. F. (1990) Molecular cloning of a human gene that is a member of the nerve growth factor family. Proc. Natl. Acad. Sci. U S A, 87, 8060-8064.

Kaplan, D. R., Hempstead, B. L., Martin, Z. D., Chao, M. V. and Parada, L. F. (1991a) The trk proto-oncogene product: a signal transducing receptor for nerve growth factor. Science, 252, 554-558.

Kaplan, D. R., Martin-Zanca, D. and Parada, L. F. (1991b) Tyrosine phosphorylation and tyrosine kinase activity of the trk proto-oncogene product induced by NGF. Nature, 350, 158-160.

Kingsley, D. M. (1994) The TGF-ß superfamily: new members, new receptors, and new genetic tests of function in different organisms. Genes & Dev., 8, 133-146.

Kishimoto, T., Akira, S. and Taga, T. (1992) Interleukin-6 and its receptor: a paradigm for cytokines. Science, 258, 593-597.

Klein, D.K., Sherman, D., Ho, W.H., Stone, D., Bennett, G.L., Moffat, B., Vandlen, R., Simmons, L., Gu, Q., Hongo, J.A., Devaux, B., Poulsen, K., Armanini, M., Nozaki, C., Asai, N., Goddard, A., Phillips, H., Henderson, C.E., Takahashi, M., Rosenthal, A. (1997) A GPI-linked protein that interacts with Ret to form a candidate neurturin receptor. Nature, 387, 717-721.

Klein, R., Conway, D., Parada, L. F. and Barbacid, M. (1990) The trkB tyrosine protein kinase gene codes for a second neurogenic receptor that lacks the catalytic kinase domain. Cell, 61, 647-656.

Klein, R., Jing, S. Q., Nanduri, V., O'Rourke, E. and Barbacid, M. (1991a) The trk proto-oncogene encodes a receptor for nerve growth factor. Cell, 65, 189-197.

Klein, R., Lamballe, F., Bryant, S. and Barbacid, M. (1992) The trkB tyrosine protein kinase is a receptor for neurotrophin-4. Neuron, 8, 947-956.

Klein, R., Nanduri, V., Jing, S. A., Lamballe, F., Tapley, P., Bryant, S., Cordon-Cardo, C., Jones, K. R., Reichardt, L. F. and Barbacid, M. (1991b) The trkB tyrosine protein kinase is a receptor for brain-derived neurotrophic factor and neurotrophin-3. Cell, 66, 395-403.

Klein, R., Parada, L. F., Coulier, F. and Barbacid, M. (1989) trkB, a novel tyrosine protein kinase receptor expressed during mouse neural development. EMBO J., 8, 3701-3709.

Klein, R., Silos, S. I., Smeyne, R. J., Lira, S. A., Brambilla, R., Bryant, S., Zhang, L., Snider, W. D. and Barbacid, M. (1994) Disruption of the neurotrophin-3 receptor gene trkC eliminates la muscle afferents and results in abnormal movements. Nature, 368, 249-251.

Klein, R., Smeyne, R. J., Wurst, W., Long, L. K., Auerbach, B. A., Joyner, A. L. and Barbacid, M. (1993) Targeted disruption of the trkB neurotrophin receptor gene results in nervous system lesions and neonatal death. Cell, 75, 113-122.

Knüsel, B., Winslow, J. W., Rosenthal, A., Burton, L. E., Seid, D. P., Nikolics, K. and Hefti, F. (1991) Promotion of central cholinergic and dopaminergic neuron differentiation by brain-derived neurotrophic factor but not neurotrophin 3. Proc Natl Acad Sci U S A, 88, 961-965.

Korsching, S. (1993) The neurotrophic factor concept: a reexamination. J. Neurosci., 13, 2739-2748.

Korsching, S., Auburger, G., Heumann, R., Scott, J. and Thoenen, H. (1985) Levels of nerve growth factor and its mRNA in the central nervous system of the rat correlate with cholinergic innervation. Embo J., 4, 1389-1393.

Korsching, S. and Thoenen, H. (1988) Developmental changes of nerve growth factor levels in sympathetic ganglia and their target organs. Dev. Biol., 126, 40-46.

Kotzbauer, P. T., Lampe, P. A., Heuckeroth, R. O., Golden, J. P., Creedon, D. J., Johnson, E. M. J. and Milbrandt, J. (1996) Neurturin, a relative of glial-cell-line-derived neurotrophic factor. Nature, 384, 467-470.

Krieglstein, K., Ruffer, M., Suter-Crazzolara, C. and Unsicker, K. (1995a) Neural functions of the transforming growth factors ß. Int. J. Dev. Neurosci., 13, 98-113.

Krieglstein, K., Suter-Crazzolara, C., Fischer, W. H. and Unsicker, K. (1995b) TGF-ß superfamily members promote survival of midbrain dopaminergic neurons and protect them against MPP+ toxicity. EMBO J., 14, 736-742.

Kushima, Y., Hama, T. and Hatanaka, H. (1992) Interleukin-6 as a neurotrophic factor for promoting the survival of cultured catecholaminergic neurons in a chemically defined medium from fetal and postnatal rat midbrains. Neurosci. Res., 13, 267-280.

Kushima, Y. and Hatanaka, H. (1992) Interleukin-6 and leukemia inhibitory factor promote the survival of acetylcholinesterase-positive neurons in culture from embryonic rat spinal cord. Neurosci. Lett., 143, 110-114.

Lamballe, F., Klein, R. and Barbacid, M. (1991) trkC, a new member of the trk family of tyrosine protein kinases, is a receptor for neurotrophin-3. Cell, 66, 967-979.

Lapchak, P. A., Jiao, S., Miller, P. J., Williams, L. R., Cummins, V., Inouye, G., Matheson, C. R. and Yan, Q. (1996) Pharmacological characterization of glial cell line-derived neurotrophic factor (GDNF): implications for GDNF as a therapeutic molecule for treating neurodegenerative diseases. Cell Tissue Res., 286, 179-189.

Large, T. H., Weskamp, G., Helder, J. C., Radeke, M. J., Misko, T. P., Shooter, E. M. and Reichardt, L. F. (1989) Structure and developmental expression of the nerve growth factor receptor in the chicken central nervous system. Neuron, 2, 1123-1134.

Lärkfors, L., Lindsay, R. M. and Alderson, R. F. (1994) Ciliary neurotrophic factor enhances the survival of purkinje cells in vitro. Eur. J. Neurosci., 6, 1015-1025.

Le Douarin, N. (1986) Cell line segregation during peripheral nervous system ontogeny. Science, 231, 1515-1522.

Le Douarin, N. M. (1980) The ontogeny of the neural crest in avian embryo chimaeras. Nature, 286, 663-669.

Le Douarin, N. M. and Teillet, M. A. (1973) The migration of neural crest cells to the wall of the digestive tract in avian embryo. J. Embryol. Exp. Morphol., 30, 31-48.

Le Douarin, N. M. and Teillet, M. A. (1974) Experimental analysis of the migration and differentiation of neuroblasts of the autonomic nervous system and of neuroectodermal mesenchymal derivatives, using a biological cell marking technique. Dev. Biol., 41, 162-184.

Lee, K. F., Li, E., Huber, L. J., Landis, S. C., Sharpe, A. H., Chao, M. V. and Jaenisch, R. (1992) Targeted mutation of the gene encoding the low affinity NGF receptor p75 leads to deficits in the peripheral sensory nervous system. Cell, 69, 737-749.

Leibrock, J., Lottspeich, F., Hohn, A., Hofer, M., Hengerer, B., Masiakowski, P., Thoenen, H. and Barde, Y. A. (1989) Molecular cloning and expression of brain-derived neurotrophic factor. Nature, 341, 149-152.

Leung, D. W., Parent, A. S., Cachianes, G., Esch, F., Coulombe, J. N., Nikolics, K., Eckenstein, F. P. and Nishi, R. (1992) Cloning, expression during development, and evidence for release of a trophic factor for ciliary ganglion neurons. Neuron, 8, 1045-1053.

Levi-Montalcini, R. (1987) The nerve growth factor 35 years later. Science, 237, 1154-1162.

Levi-Montalcini, R. and Hamburger, V. (1951) Selective growth-stimulating effects of mouse sarcoma on the sensory and sympathetic nervous system of the chick embryo. J. Exp. Zool., 116, 321-361.

Lewis, M. E., Neff, N. T., Contreras, P. C., Strong, D. B., Oppenheim, R. W., Grebow, P. E. and Vaught, J. L. (1993) Insulin-like growth factor-I: potential for treatment of motor neuronal disorders. Exp. Neurol., 124, 73-88.

Li, L., Oppenheim, R. W., Lei, M. and Houenou, L. J. (1994) Neurotrophic agents prevent motoneuron death following sciatic nerve section in the neonatal mouse. J. Neurobiol., 25, 759-766.

Li, L., Wu, W., Lin, L. F. H., Lei, M., Oppenheim, R. W. and Houenou, L. J. (1995) Rescue of adult mouse motoneurons from injury-induced cell death by glial cell line-derived neurotrophic factor. Proc. Natl. Acad. Sci. USA, 92, 9771-9775.

Lin, H. Y. and Lodish, H. F. (1993) Receptors for the TGF-ß superfamily: multiple polypeptides and serine/threonine kinases. Trends Cell Biol., 3, 14-19.

Lin, H. Y., Wang, X. F., Ng-Eaton, E., Weinberg, R. A. and Lodish, H. F. (1992) Expression cloning of the TGF-ß type II receptor, a functional transmembrane serine/threonine kinase. Cell, 68, 775-785.

Lin, L. F. H., Doherty, D. H., Lile, J. D., Bektesh, S. and Collins, F. (1993) GDNF: A glial cell line-derived neurotrophic factor for midbrain dopaminergic neurons. Science, 260, 1130-1132.

Lin, L. F. H., Mismer, D., Lile, J. D., Armes, L. G., Butler, E. T., Vannice, J. L. and Collins, F. (1989) Purification, cloning, and expression of ciliary neurotrophic factor (CNTF). Science, 246, 1023-1025.

Lin, L. F. H., Zhang, T. J., Collins, F. and Armes, L. G. (1994) Purification and initial characterization of rat B49 glial cell line-derived neurotrophic factor. J. Neurochem., 63, 758-768.

Lindsay, R. M., Thoenen, H. and Barde, Y. A. (1985) Placode and neural crest-derived sensory neurons are responsive at early developmental stages to brain-derived neurotrophic factor. Devel. Biol., 112, 319-328.

Liu, F., Hata, A., Baker, J., Doody, J., Carcamo, J., Harland, R. and Massagué, J. (1996) A human Mad protein acting as a BMP-regulated transcriptional activator. Nature, 381, 620-623.

Liu, X., Ernfors, P., Wu, H. and Jaenisch, R. (1995) Sensory but not motor neuron deficits in mice lacking NT4 and BDNF. Nature, 375, 238-241.

Lorenzo, M. J., Gish, G. D., Houghton, C., Stonehouse, T. J., Pawson, T., Ponder, B. A. J. and Smith, D. P. (1997) Ret alternative splicing influences the interaction of activated Ret with the SH2 and PTB domains of Shc, and the SH2 domain of Grb2. Oncogene, 14, 763-771.

Maisonpierre, P. C., Belluscio, L., Friedman, B., Alderson, R. F., Wiegand, S. J., Furth, M. E., Lindsay, R. M. and Yancopoulos, G. D. (1990a) NT-3, BDNF, and NGF in the developing rat nervous system: parallel as well as reciprocal patterns of expression. Neuron, 5, 501-509.

Maisonpierre, P. C., Belluscio, L., Squinto, S., Ip, N. Y., Furth, M. E., Lindsay, R. M. and Yancopoulos, G. D. (1990b) Neurotrophin-3: a neurotrophic factor related to NGF and BDNF. Science, 247, 1446-1451.

Maisonpierre, P. C., Le Beau, M., Espinosa, R., Ip, N. Y., Belluscio, L., de la Monte, S., Squinto, S., Furth, M. E. and Yancopoulos, G. D. (1991) Human and rat brain-derived neurotrophic factor and neurotrophin-3: gene structures, distributions, and chromosomal localizations. Genomics, 10, 558-568.

Mak, Y. F. and Ponder, B. A. J. (1996) RET oncogene. Curr. Opin. Genet. Dev., 6, 82-86.

Malik, N., Kallestad, J. C., Gunderson, N. L., Austin, S. D., Neubauer, M. G., Ochs, V., Marquardt, H., Zarling, J. M., Shoyab, M., Wei, C. M., Linsley, P. S. and Rose, T. M. (1989) Molecular cloning, sequence analysis, and functional expression of a novel growth regulator, Oncostatin M. Mol. Cell. Biol., 9, 2847-2853.

Manthorpe, M., Skaper, S. D., Williams, L. R. and Varon, S. (1986) Purification of adult rat sciatic nerve ciliary neuronotrophic factor. Brain Res., 367, 282-286.

Manthorpe, M. and Varon, S. (1985) In Guroff, G. (ed.), Growth and maturation factors. Wiley, New York, pp. 77-117.

Marcos-Gutierrez, C. V., Wilson, S. W., Holder, N. and Pachnis, V. (1997) The zebrafish homologue of the Ret receptor and its pattern of expression during embryogenesis. Oncogene, 14, 879-889.

Martin-Zanca, D., Hughes, S. H. and Barbacid, M. (1986) A human oncogene formed by the fusion of truncated tropomyosin and protein tyrosine kinase sequences. Nature, 319, 743-748.

Martin-Zanca, D., Oskam, R., Mitra, C., Copeland, T. and Barbacid, M. (1989) Molecular and biochemical characterisation of the human trk proto-oncogene. Mol. Cell. Biol., 9, 24-33.

Martinou, J. C., Le Van Thai, A., Valette, A. and Weber, M. J. (1990) Transforming growth factor b1 is a potent survival factor for rat embryo motoneurons in culture. Dev. Brain Res., 52, 175-181.

Martinou, J. C., Martinou, I. and Kato, A. C. (1992) Cholinergic differentiation factor (CDF/LIF) promotes survival of isolated rat embryonic motoneurons in vitro. Neuron, 8, 737-744.

Martins, L. M. and Earnshaw, W. (1997) Apoptosis: alive and kicking in 1997. Trends Cell. Biol., 7, 111-114.

Mason, I. (1996) The GDNF receptor: recent progress and unanswered questions. Mol. Cell. Neurosci., 8, 112-119.

Massagué, J. (1990) The transforming growth factor-ß family. Annu. Rev. Cell Biol., 6, 597-641.

Massagué, J. (1992) Receptors for the TGF-ß family. Cell, 69, 1067-1070.

Massagué, J. (1996) TGFß signaling: receptors, transducers, and Mad proteins. Cell, 85, 947-950.

Massagué, J. (1996b) Crossing receptor boundaries. Nature, 382, 29-30.

Massagué, J., Attisano, L. and Wrana, J. L. (1994) The TGF-ß family and its composite receptors. Trends Cell Biol., 4, 172-178.

Masu, Y., Wolf, E., Holtmann, B., Sendtner, M., Brem, G. and Thoenen, H. (1993) Disruption of the CNTF gene results in motor neuron degeneration. Nature, 365, 27-32.

Mathews, L. S. and Vale, W. W. (1991) Expression cloning of an activin receptor, a predicted transmembrane serine kinase. Cell, 65, 973-982.

Mathews, L. S., Vale, W. W. and Kintner, C. R. (1992) Cloning of a second type of activin receptor and functional characterization in Xenopus embryos. Science, 255, 1702-1705.

McDonald, N. Q. and Hendrickson, W. A. (1993) A structural superfamily of growth factors containing a cysteine knot motif. Cell, 73, 421-424.

McDonald, N. Q., Lapatto, R., Murray, R. J., Gunning, J., Wlodawer, A. and Blundell, T. L. (1991) New protein fold revealed by a 2.3-A resolution crystal structure of nerve growth factor. Nature, 354, 411-414.

Meakin, S. O. and Shooter, E. M. (1992) The nerve growth factor family of receptors. Trends Neurosci., 15, 323-331.

Meier, R., Becker, A. M., Gotz, R., Heumann, R., Shaw, A. and Thoenen, H. (1986) Molecular cloning of bovine and chick nerve growth factor (NGF): delineation of conserved and unconserved domains and their relationship to the biological activity and antigenicity of NGF. Embo J., 5, 1489-1493.

Middlemas, D. S., Lindberg, R. A. and Hunter, T. (1991) trkB, a neural receptor protein-tyrosine kinase: evidence for a full-length and two truncated receptors. Mol. Cell. Biol., 11, 143-153.

Moore, M. W., Klein, R. D., Farinas, I., Sauer, H., Armanini, M., Phillips, H., Reicherdt, L. F., Ryan, A. M., Carver-Moore, K. and Rosenthal, A. (1996) Renal and neuronal abnormalities in mice lacking GDNF. Nature, 382, 76-79.

Mount, H. T. J., Dean, D. O., Alberch, J., Dreyfus, C. F. and Black, I. B. (1995) Glial cell line-derived neurotrophic factor promotes the survival and morphologic differentiation of Purkinje cells. Proc. Natl. Acad. USA, 92, 9092-9096.

Mulligan, L. M., Eng, C., Healey, C. S., Clayton, D., Kwok, J. B. J., Gardner, E., Ponder, M. A., Frilling, A., Jackson, C. E., Lehnert, H., Neumann, H. P. H., Thibodeau, S. N. and Ponder, B. A. J. (1994) Specific mutations of the Ret proto-oncogene are related to disease phenotype in MEN 2A and FMTC. Nature Genetics, 6, 70-74.

Mulligan, L. M., Kwok, J. B. J., Healy, C. S., Elsdon, M. J., Eng, C., Gardner, E., Love, D. R., Mole, S. E., Moore, J. K., Papi, L., Ponder, M. A., Telenius, H., Tunnacliffe, A. and Ponder, B. A. J. (1993) Germ-line mutations of the RET proto-oncogene in multiple endocrine neoplasia type 2A. Nature, 363, 458-460.

Murakami, M., Hibi, M., Nakagawa, N., Nakagawa, T., Yasukawa, K., Yamanishi, K., Taga, T. and Kishimoto, T. (1993) IL-6-induced homodimerization of gp130 and associated activation of a tyrosine kinase. Science, 260, 1808-1810.

Murphy, M., Reid, K., Brown, M. A. and Bartlett, P. F. (1993) Involvement of leukemia inhibitory factor and nerve growth factor in the development of dorsal root ganglion neurons. Development, 117, 1173-1182.

Murphy, P. G., Grondin, J., Altares, M. and Richardson, P. M. (1995) Induction of interleukin-6 in axotomized neurons. J. Neurosci., 15, 5130-5138.

Nagata, S. and Golstein, P. (1995) The Fas death factor. Science, 267, 1449-1456.

Nawa, H., Nakanishi, S. and Patterson, P. H. (1991) Recombinant cholinergic differentiation factor (leukemia inhibitory factor) regulates sympathetic neuron phenotype by alterations in the size and amounts of neuropeptide mRNAs. J. Neurochem., 56, 2147-2150.

Nellen, D., Affolter, M. and Basler, K. (1994) Receptor serine/threonine kinases implicated in the control of Drosophila body pattern by decapentaplegic. Cell, 78, 225-237.

Ninkina, N., Graschuck, M., Buchman, V. L. and Davies, A. M. (1997) TrkB variants with deletions in the leucine-rich motifs of the extracellular domain. J. Biol. Chem., 272, 13019-13025.

Nishi, R. and Berg, D. K. (1981) Two components from eye tissue that differentially stimulate the growth and development of ciliary ganglion neurons in cell culture. J. Neurosci., 1, 505-513.

Noden, D. M. (1978) The control of avian cephalic neural crest cytodifferentiation 11. Neural tissues. Dev. Biol., 67, 313-329.

Nosrat, C. A., Tomac, A., Lindqvist, E., Lindskog, S., Humpel, C., Strömberg, I., Ebendal, T., Hoffer, B. J. and Olson, L. (1996) Cellular expression of GDNF mRNA suggests multiple functions inside and outside the nervous system. Cell Tissue Res., 286, 191-207.

Olson, L. (1994) Neurotrophins in neurodegenerative disease: theoretical issues and clinical trials. Neurochemistry, 25, 1-3.

Oppenheim, R. W. (1991) Cell death during development of the nervous system. Ann. Rev. Neurosci., 14, 453-501.

Oppenheim, R. W., Houenou, L. J., Johnson, J. E., Lin, L. F. H., Li, L., Lo, A. C., Newsome, A. L., Prevette, D. M. and Wang, S. (1995) Developing motoneurons rescued from programmed and axotomy-induced cell death by GDNF. Nature, 373, 344-346.

Oppenheim, R. W., Prevette, D., Yin, Q. W., Collins, F. and MacDonald, J. (1991) Control of embryonic motoneuron survival in vivo by ciliary neurotrophic factor. Science, 251, 1616-1618.

Oppenheim, R. W., Qin-Wei, Y., Prevette, D. and Yan, Q. (1992) Brain-derived neurotrophic factor rescues developing avian motoneurons from cell death. Nature, 360, 755-757.

Pachnis, V., Mankoo, B. and Costantini, F. (1993) Expression of the c-ret proto-oncogene during mouse embryogenesis. Development, 119, 1005-1017.

Pandey, A., Duan, H., DiFiore, P. P. and Dixit, V. M. (1995) The Ret receptor protein tyrosine kinase associates with the SH2-containing adapter protein Grb10. J. Biol. Chem., 270, 21461-21463.

Pasini, B., Ceccherini, I. and Romeo, G. (1996) RET mutations in human disease. Trends Genet., 12, 138-144.

Patterson, P. H. (1994) Leukemia inhibitory factor, a cytokine at the interface between neurobiology and immunology. Proc. Natl. Acad. Sci. USA, 91, 7833-7835.

Pennica, D., King, K. L., Shaw, K. J., Luis, E., Rullamas, J., Luoh, S. M., Darbonne, W. C., Knutzon, D. S., Yen, R., Chien, K. R., Barker, J. B. and Wood, W. I. (1995a) Expression cloning of cardiotrophin-1, a cytokine that induces cardiac myocyte hypertrophy. Proc. Natl. Acad. Sci. USA, 92, 1142-1146.

Pennica, D., Shaw, K. J., Swanson, T. A., Moore, M. W., Shelton, D., Zioncheck, K. A., Rosenthal, A., Taga, T., Paoni, N. F. and Wood, W. I. (1995b) Cardiotrophin-1. Biological activities and binding to the leukemia inhibitory factor receptor/gp130 signaling complex. J. Biol. Chem., 270, 10915-10922.

Penton, A., Chen, Y., Staehling-Hampton, K., Wrana, J. L., Attisano, L., Szidonya, J., Cassill, J. A., Massagué, J. and Hoffmann, F. M. (1994) Identification of two bone morphogenetic protein type I receptors in Drosophila and evidence that Brk25D is a decapentaplegic receptor. Cell, 78, 239-250.

Pichel, J. G., Shen, L., Sheng, H. Z., Granholm, A. C., Drago, J., Grinberg, A., Lee, E. J., Huang, S. P., Saarma, M., Hoffer, B. J., Sariola, H. and Westphal, H. (1996) Defects in enteric innervation and kidney development in mice lacking GDNF. Nature, 382, 73-76.

Pinon, L. G. P., Minichiello, L., Klein, R. and Davies, A. M. (1996) Timing of neuronal death in trkA, trkB and trkC mutant embryos reveals developmental changes in sensory neuron dependence on Trk signalling. Development, 122, 3255-3261.

Pinon, L. G. P., Robinson, M. and Davies, A. M. (1995) High specificity of neurotrophins in the embryonic chicken trigeminal system. Euro. J. Neurosci., 7, 2397-2402.

Poulsen, K. T., Armanini, M. P., Klein, R. D., Hynes, M. A., Phillips, H. S. and Rosenthal, A. (1994) TGFß2 and TGFß3 are potent survival factors for midbrain dopaminergic neurons. Neuron, 13, 1245-1252.

Puffenberger, E. G., Hosoda, K., Washington, S. S., Nakao, K., deWit, D., Yanagisawa, M. and Chakravarti, A. (1994) A missense mutation of the endothelin-B receptor gene in multigenic Hirschsprung's disease. Cell, 79, 1257-1266.

Radeke, M. J., Misko, T. P., Hsu, C., Herzenberg, L. A. and Shooter, E. M. (1987) Gene transfer and molecular cloning of the rat nerve growth factor receptor. Nature, 325, 593-597.

Radziejewski, C., Robinson, R. C., DiStefano, P. and Taylor, J. W. (1992) Dimeric structure and conformational stability of brain-derived neurotrophic factor and neurotrophin-3. Biochemistry, 31, 4431-4436.

Raftery, L. A., Twobly, V., Wharton, K. and Gelbart, W. M. (1995) Genetic screens to identify elements of the decapentaplegic signaling pathway in Drosophila. Genetics, 139, 241-254.

Rao, L. and White, E. (1997) Bcl-2 and the ICE family of apoptotic regulators: making a connection. Curr. Op. Genet. Dev., 7, 52-58.

Reichert, F., Levitzky, R. and Rotshenker, S. (1996) Interleukin 6 in intact and injured mouse peripheral nerves. Eur. J. Neurosci., 8, 530-535.

Richards, L. J., Kilpatrick, T. J., Dulton, R., Tan, S. S., Gearing, D. P., Bartlett, P. F. and Murphy, M. (1996) Leukaemia inhibitory factor or related factors promote the differentiation of neuronal and astrocytic precursors within the developing murine spinal cord. Eur. J. Neurosci., 8, 291-299.

Roberts, A. B. and Sporn, M. B. (1990) The transforming growth factor-ßs. Handbook Exp. Pharmacol., 95, 419-472.

Robertson, K. and Mason, I. (1997) The GDNF-RET signalling partnership. Trends Genet., 13, 1-3.

Robinson, M., Buj-Bello, A. and Davies, A. M. (1996) Paracrine interactions of BDNF involving NGF-dependent embryonic sensory neurons. Mol. Cell. Neurosci., 7, 143-151.

Rohrer, H. and Thoenen, H. (1987) Relationship between differentiation and terminal mitosis: chick sensory and ciliary neurons differentiate after terminal mitosis of precursor cells, whereas sympathetic neurons continue to divide after differentiation. J. Neurosci., 7, 3739-3748.

Romeo, G., Ronchetto, P., Luo, Y., Barone, V., Seri, M., Ceccherimi, I., Pasini, B., Bocciardi, R., Lerone, M., Kääriäinen, H. and Martucciello, G. (1994) Point mutations affecting the tyrosine kinase domain of the RET proto-oncogene in Hirschsprung's disease. Nature, 367, 377-378.

Rosen, V. and Thies, R. S. (1992) The BMP proteins in bone formation and repair. Trends Genet., 8, 97-102.

Rosenthal, A., Goeddel, D. V., Nguyen, T., Lewis, M., Shih, A., Laramee, G. R., Nikolics, K. and Winslow, J. W. (1990) Primary structure and biological activity of a novel human neurotrophic factor. Neuron, 4, 767-773.

Rothman, T. P., Gershon, M. D. and Holtzer, H. (1978) The relationship of cell division to the acquistion of adrenergic characteristics by developing sympathetic ganglion cell precursors. Devel. Biol., 65, 322-341.

Ruberte, E., Marty, T., Nellen, D., Affolter, M. and Basler, K. (1995) An absolute requirement for both the type II and type I receptors, Punt and Thick veins, for Dpp signalling in vivo. Cell, 80, 889-897.

Saadat, S., Sendtner, M. and Rohrer, H. (1989) Ciliary neurotrophic factor induces cholinergic differentiation of rat sympathetic neurons in culture. J. Cell Biol., 108, 1807-1816.

Sagot, Y., Tan, S. A., Hammang, J. P., Aebischer, P. and Kato, A. C. (1996) GDNF slows loss of motoneurons but not axonal degeneration or premature death of pmn/pmn mice. J. Neurosci., 16, 2335-2341.

Salomon, R., Attié, T., Pelet, A., Bidaud, C., Eng, C., Amiel, J., Sarnacki, S., Goulet, O., Ricour, C., Nihoul-Fekete, C., Munnich, A. and Lyonnet, S. (1996) Germline mutations of the RET ligand GDNF are not suficient to cause Hirschsprung disease. Nat. Genet., 14, 345-347.

Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) Molecular cloning : a laboratory manual. In Harbor, C. S. (Ed.), New York: Cold Spring Harbor Laboratory Press.

Sanchez, M. P., Silos-Santiago, I., Frisén, J., He, B., Sergio, S. A. and Barbacid, M. (1996) Renal agenesis and the absence of enteric neurons in mice lacking GDNF. Nature, 382, 70-73.

Sanger, F., Nicklen, S. and Coulson, A. R. (1977) DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA, 74, 5463-5467.

Santoro, M., Carlomagno, F., Romano, A., Bottaro, D. P., Dathan, N. A., Grieco, M., Fusco, A., Vecchio, G., Matoskova, B., Kraus, M. H. and DiFiore, P. P. (1995) Activation of RET as a dominant transforming gene by germline mutations of MEN2A and MEN2B. Science, 267, 381-383.

Santoro, M., Dathan, N. A., Berlingieri, M. T., Bongarzone, I., Paulin, C., Grieco, M., Pierotti, M. A., Vecchio, G. and Fusco, A. (1994) Molecular characterization of RET/PTC3: a novel rearranged version of the RET proto-oncogene in a human thyroid papillary carcinoma. Oncogene, 9, 509-516.

Sato, N. and Miyajima, A. (1994) Multimeric cytokine receptors: common versus specific functions. Curr. Opin. Cell Biol., 6, 174-179.

Sauer, H., Rosenblad, C. and Björklund, A. (1995) Glial cell line-derived neurotrophic factor but not transforming growth factor ß3 prevents delayed degeneration of nigral dopaminergic neurons following striatal 6-hydroxydopamine lesion. Proc. Natl. Acad. Sci. USA, 92, 8935-8939.

Saxen, L. (1987) Organogenesis of the kidney. Cambridge: Cambridge University Press.

Schaar, D. G., Sieber, B. A., Dreyfus, C. F. and Black, I. B. (1993) Regional and cell-specific expression of GDNF in rat brain. Exp. Neurol., 124, 368-371.

Schaar, D. G., Sieber, B. A., Sherwood, A. C., Dean, D., Mendoza, G., Ramakrishnan, L., Dreyfus, C. F. and Black, I. B. (1994) Multiple astrocyte transcripts encode nigral trophic factors in rat and human. Exp. Neurol., 130, 387-393.

Schecterson, L. and Bothwell, M. (1992) Novel roles for neurotrophins are suggested by BDNF and NT-3 mRNA expression in developing neurons. Neuron, 9, 449-463.

Schindelhauer, D., Schuffenhauer, S., Gasser, T., Steinkasserer, A. and Meitinger, T. (1995) The gene coding for glial cell line derived neurotrophic factor (GDNF) maps to chromosome 5p12-p13.1. Genomics, 28, 605-607.

Schlunegger, M. P. and Grütter, M. G. (1992) An unusual feature revealed by the crystal structure at 2.2 A resolution of human transforming growth factor ß-2. Nature, 358, 430-434.

Schuchardt, A., D'Agati, V., Larsson-Blomberg, L., Costantini, F. and Pachnis, V. (1994) Defects in the kidney and enteric nervous system of mice lacking the tyrosine kinase receptor Ret. Nature, 367, 380-383.

Schuchardt, A., Srivinas, S., Pachnis, V. and Costantini, F. (1995) Isolation and characterization of a chicken homolog of the c-ret proto-oncogene. Oncogene, 10, 641-649.

Scott, J., Selby, M., Urdea, M., Quiroga, M., Bell, G. I. and Rutter, W. J. (1983) Isolation and nucleotide sequence of a cDNA encoding the precursor of mouse nerve growth factor. Nature, 302, 538-540.

Segal, R. A. and Greenberg, M. E. (1996) Intracellular signalling pathways activated by neurotrophic factors. Annu. Rev. Neurosci., 19, 463-489.

Sekelsky, J. J., Newfeld, S. J., Raftery, L. A., Chartoff, E. H. and Gelbart, W. M. (1995) Genetic characterization and cloning of Mothers against dpp, a gene required for decapentaplegic function in Drosophila melanogaster. Genetics, 139, 1347-1358.

Sendtner, M., Carroll, P., Holtmann, B., Hughes, R. A. and Thoenen, H. (1994) Ciliary neurotrophic factor. J. Neurobiol., 25, 1436-1453.

Sendtner, M., Kreutzberg, G. W. and Thoenen, H. (1990) Ciliary neurotrophic factor prevents the degeneration of motor neurons after axotomy. Nature, 345, 440-441.

Sendtner, M., Schmalbruch, H., Stöckli, K. A., Caroll, P., Kreutzberg, G. W. and Thoenen, H. (1992b) Ciliary neurotrophic fector prevents degeneration of motor neurons in mouse mutant progressive motor neuronopathy. Nature, 358, 502-504.

Sendtner, M., Stöckli, K. A. and Thoenen, H. (1992a) Synthesis and localization of ciliary neurotrophic factor in the sciatic nerve of the adult rat after lesion and during regeneration. J. Cell Biol., 118, 139-148.

Serbedzija, G. N., Burgan, S., Fraser, S. E. and Bronner-Fraser, M. (1991) Vital dye labeling demonstrates a sacral neural crest contribution to the enteric nervous system of chick and mouse embryos. Development, 111, 857-866.

Sheng, Z., Pennica, D., Wood, W. I. and Chien, K. R. (1996) Cardiotrophin-1 displays early expression in the murine heart tube and promotes cardiac myocyte survival. Development, 122, 419-428.

Shults, C. W., Kimber, T. and Martin, D. (1996) Intrastriatal injection of GDNF attenuates the effects of 6-hydroxydopamine. Neuroreport, 7, 627-631.

Smeyne, R. J., Klein, R., Schnapp, A., Long, L. K., Bryant, S., Lewin, A., Lira, S. A. and Barbacid, M. (1994) Severe sensory and sympathetic neuropathies in mice carrying a disrupted Trk/NGF receptor gene. Nature, 368, 246-249.

Snider, W. D. (1994) Functions of the neurotrophins during nervous system development: what the knockouts are teaching us. Cell, 77, 627-638.

Soppet, D., Escandon, E., Maragos, J., Middlemas, D. S., Reid, S. W., Blair, J., Burton, L. E., Stanton, B. R., Kaplan, D. R., Hunter, T., Nikolics, K. and Parada, L. F. (1991) The neurotrophic factors brain-derived neurotrophic factor and neurotrophin-3 are ligands for the trkB tyrosine kinase receptor. Cell, 65, 895-903.

Sporn, M. B. and Roberts, A. B. (1992) Transforming growth factors-ß: recent progress and new challenges. J. Cell. Biol., 119, 1017-1021.

Springer, J. E., Mu, X., Bergmann, L. W. and Trojanowski, J. Q. (1994) Expression of GDNF mRNA in rat and human nervous tissue. Exp. Neurol., 127, 167-170.

Squinto, S. P., Stitt, T. N., Aldrich, T. H., Davis, S., Bianco, S. M., Radziejewski, C., Glass, D. J., Masiakowski, P., Furth, M. E., Valenzuela, D. M., DiStefano, P. S. and Yancopoulos, G. D. (1991) trkB encodes a functional receptor for brain-derived neurotrophic factor and neurotrophin-3 but not nerve growth factor. Cell, 65, 885-893.

Stahl, N., Boulton, T. G., Farruggella, T., Ip, N. Y., Davis, S., Witthuhn, B. A., Quelle, F. W., Silvennoinen, O., Barbieri, G., Pellegrini, S., Ihle, J. N. and Yancopoulos, G. D. (1994) Association and activation of Jak-Tyk kinases by CNTF-LIF-OSM-IL-6 ß receptor components. Science, 263, 92-95.

Stahl, N. and Yancopoulos, G. D. (1993) The alphas, betas, and kinases of cytokine receptor complexes. Cell, 74, 587-590.

Stahl, N. and Yancopoulos, G. D. (1994) The tripartite CNTF receptor complex: activation and signaling involves components shared with other cytokines. J. Neurobiol., 25, 1454-1466.

Stewart, C. L., Kasper, P., Brunet, L. J., Bhatt, H., Gadi, I., Kontgen, F. and Abbondanzo, S. J. (1992) Blastocyst implantation depends on maternal expression of leukemia inhibitory factor. Nature, 359, 76-79.

Stöckli, K. A., Lillien, L. E., Näher-Noé, M., Breitfeld, G., Hughes, R. A., Raff, M. C., Thoenen, H. and Sendtner, M. (1991) Regional distribution, developmental changes, and cellular localization of CNTF-mRNA and protein in the rat brain. J. Cell Biol., 115, 447-459.

Stöckli, K. A., Lottspeich, F., Sendtner, M., Masiakowski, P., Carroll, P., Götz, R., Lindholm, D. and Thoenen, H. (1989) Molecular cloning, expression and regional distribution of rat ciliary neurotrophic factor. Nature, 342, 920-923.

Strömberg, I., Björklund, L., Johansson, M., Tomac, A., Collins, F., Olson, L., Hoffer, B. and Humpel, C. (1993) Glial cell line-derived neurotrophic factor is expressed in the developing but not adult striatum and stimulates developing dopamine neurons in vivo. Exp. Neurol., 124, 401-412.

Suter-Crazzolara, C. and Unsicker, K. (1994) GDNF is expressed in many tissues outside the CNS. Neuroreport, 5, 2486-2488.

Sutter, A., Riopelle, R. J., Harris-Warrick, R. M. and Shooter, E. M. (1979) Nerve growth factor receptors. Characterization of two distinct classes of binding sites on chick embryo sensory ganglia. J. Biol. Chem., 254, 5972-5982.

Suvanto, P., Hiltunen, J. O., Arumäe, U., Moshnyakov, M., Sariola, H., Sainio, K. and Saarma, M. (1996) Localization of glial cell line-derived neurotrophic factor (GDNF) mRNA in embryonic rat by in situ hybridisation. Eur. J. Neurosci., 8, 816-822.

Taga, T., Hibi, M., Hirata, Y., Yamasaki, K., Yasukawa, K., Matsuda, T., Hirano, T. and Kishimoto, T. (1989) Interleukin-6 triggers the association of its receptor with a possible signal transducer, gp130. Cell, 58, 573-581.

Tahira, T., Ishizaka, Y., Itoh, F., Sugimura, T. and Nagao, M. (1990) Characterization of ret proto-oncogene mRNAs encoding two isoforms of the protein product in a human neuroblastoma cell line. Oncogene, 5, 97-102.

Takahashi, M., Buma, Y. and Hiai, H. (1989) Isolation of ret proto-oncogene cDNA with an amino-terminal signal sequence. Oncogene, 4, 805-806.

Takahashi, M., Buma, Y., Iwamoto, T., Inaguma, Y., Ikeda, H. and Hiai, H. (1988) Cloning and expression of the ret proto-oncogene encoding a tyrosine kinase with two potential transmembrane domains. Oncogene, 3, 571-578.

Takahashi, M. and Cooper, G. M. (1987) Ret transforming gene encodes a fusion protein homologous to tyrosine kinases. Mol. Cell Biol., 7, 1378-1385.

Takahashi, M., Ritz, J. and Cooper, G. M. (1985) Activation of a novel human transforming gene, ret, by DNA rearrangement. Cell, 42, 581-588.

Thoenen, H. (1995) Neurotrophins and neuronal plasticity. Science, 270, 593-598.

Timmusk, T., Belluardo, N., Metsis, M. and Persson, H. (1993) Widespread and developmentally regulated expression of neurotrophin-4 mRNA in rat brain and peripheral tissues. Eur. J. Neurosci., 5, 605-613.

Tomac, A., Lindqvist, E., Lin, L. F. H., Ogren, S. O., Young, D., Hoffer, B. J. and Olson, L. (1995b) Protection and repair of the nigrostriatal dopaminergic system by GDNF in vivo. Nature, 373, 335-339.

Tomac, A., Widenfalk, J., Lin, L. F. H., Kohno, T., Ebendal, T., Hoffer, B. J. and Olson, L. (1995a) Retrograde axonal transport of glial cell line-derived neurotrophic factor in the adult nigrostriatal system suggests a trophic role in the adult. Proc. Natl. Acad. Sci. USA, 92, 8274-8278.

Treanor, J. J. S., Goodman, L., de Sauvage, F., Stone, D.M., Poulsen, K.T., Beck, C. D., Gray, C., Armanini, M.P., Pollock, R.A., Hefti, F., Phillips, H.S., Goddard, A., Moore, M.W., Buj-Bello, A., Davies, A.M., Asai, N., Takahashi, M., Vandlen, R., Henderson, C.E. and Rosenthal, A. (1996) Characterization of a multicomponent receptor for GDNF. Nature, 382, 80-83.

Trupp, M., Arenas, E., Fainzilber, M., Nilsson, A. S., Sieber, B. A., Grigoriou, M., Kilkenny, C., Salazar-Grueso, E., Pachnis, V., Arumae, U., Sariola, H., Saarma, M. and Ibanez, C. F. (1996) Functional receptor for GDNF encoded by the c-ret proto-oncogene. Nature, 381, 785-789.

Trupp, M., Ryden, M., Jörnvall, H., Funakoshi, H., Timmusk, T., Arenas, E. and Ibanez, C. F. (1995) Peripheral expression and biological activities of GDNF, a new neurotrophic factor for avian and mammalian peripheral neurons. J. Cell Biol., 130, 137-148.

Tsoulfas, P., Soppet, D., Escandon, E., Tessarollo, L., Mendoza, R. J., Rosenthal, A., Nikolics, K. and Parada, L. F. (1993) The rat trkC locus encodes multiple neurogenic receptors that exhibit differential response to neurotrophin-3 in PC12 cells. Neuron, 10, 975-990.

Tsujimoto, Y., Gorham, J., Cossman, J., Jaffe, E. and Croce, C. M. (1985) The t(14;18) chromosome translocations involved in B-cell neoplasms result from mistakes in VDJ joining. Science, 229, 1390-1393.

Tsuzuki, T., Takahashi, M., Asai, N., Iwashita, T., Matsuyama, M. and Asai, J. (1995) Spatial and temporal expression of the ret proto-oncogene product in embryonic, infant and adult rat tissues. Oncogene, 10, 191-198.

Ullrich, A., Gray, A., Berman, C. and Dull, T. J. (1983) Human ß-nerve growth factor gene sequence highly homologous to that of mouse. Nature, 303, 821-825.

Unsicker, K., Meier, C., Krieglstein, K., Sartor, B. M. and Flanders, K. C. (1996) Expression, localization, and function of transforming growth factor-ßs in embryonic chick spinal cord, hindbrain, and dorsal root ganglia. J. Neurobiol., 29, 262-276.

Valenzuela, D. M., Maisonpierre, P. C., Glass, D. J., Rojas, E., Nunez, L., Kong, Y., Gies, D. R., Stitt, T. N., Ip, N. Y. and Yancopoulos, G. D. (1993) Alternative forms of rat TrkC with different functional capabilities. Neuron, 10, 963-974.

Vaux, D. L., Cory, S. and Adams, J. M. (1988) Bcl-2 gene promotes the survival of haemopoietic cells and cooperates with c-myc to immortalize pre-B cells. Nature, 335, 440-442.

Vega, Q. C., Worby, C. A., Lechner, M. S., Dixon, J. E. and Dressler, G. R. (1996) Glial cell line-derived neurotrophic factor activates the receptor tyrosine kinase RET and promotes kidney morphogenesis. Proc. Natl. Acad. Sci. USA, 93, 10657-10661.

Vejsada, R., Sagot, Y. and Kato, A. C. (1995) Quantitative comparison of the transient rescue effects of neurotrophic factors on axotomized motoneurons in vivo. Eur. J. Neurosci., 7, 108-115.

Vogel, K. S. (1992) Origins and early development of vertebrate cranial sensory neurons. Oxford University Press, New York.

Wall, N. A. and Hogan, B. L. M. (1994) TGF-ß related genes in development. Curr. Op. Gen. & Dev., 4, 517-522.

Wang, X. F., Lin, H. Y., Ng-Eaton, E., Downward, J., Lodish, H. F. and Weinberg, R. A. (1991) Expression cloning and characterization of the TGF-ß type III receptor. Cell, 67, 797-805.

Welcher, A. A., Bitler, C. M., Radeke, M. J. and Shooter, E. M. (1991) Nerve growth factor binding domain of the nerve growth factor receptor. Proc. Natl. Acad. Sci. USA, 88, 159-163.

Whittemore, S. R., Ebendal, T., Lärkfors, L., Olson, L., Seiger, A., Strömberg, I. and Persson, H. (1986) Development and regional expression of ß nerve growth factor messenger RNA and protein in the rat central nervous system. Proc. Natl. Acad. Sci. USA, 83, 817-821.

Wieser, R., Wrana, J. L. and Massagué, J. (1995) GS domain mutations that constitutively activate TßR-I, the downstream signaling component in the TGF-ß receptor complex. EMBO J., 14, 2199-2208.

Williams, L. R., Inouye, G., Cummins, V. and Pelleymounter, M. A. (1996) Glial cell line-derived neurotrophic factor sustains axotomized basal forebrain cholinergic neurons in vivo: dose-response comparison to nerve growth factor and brain-derived neurotrophic factor. J. Pharm. Exp. Therap., 277, 1140-1151.

Worby, C. A., Vega, Q. C., Zhao, Y., Chao, H. H. J., Seasholtz, A. F. and Dixon, J. E. (1996) Glial cell line-derived neurotrophic factor signals through the RET receptor and activates mitogen-activated protein kinase. J. Biol. Chem., 271, 23619-23622.

Wrana, J. L., Attisano, L., Wieser, R., Ventura, F. and Massagué, J. (1994) Mechanism of activation of the TGF-ß receptor. Nature, 370, 341-347.

Wright, D. E. and Snider, W. D. (1996) Focal expression of glial cell line-derived neurotrophic factor in developing mouse limb bud. Cell Tissue Res., 286, 209-217.

Wyllie, A. H., Kerr, J. F. R. and Currie, A. R. (1980) Cell death: the significance of apoptosis. Int. Rev. Cytol., 68, 251-306.

Xie, T., Finelli, A. and Padgett, R. W. (1994) The Drosophila saxophone gene: a serine-threonine kinase receptor of the TGFß superfamily. Science, 263, 1756-1759.

Yamamori, T., Fukada, K., Aebersold, R., Korsching, S., Fann, M. J. and Patterson, P. H. (1989) The cholinergic neuronal differentiation factor from heart cells is identical to leukemia inhibitory factor. Science, 246, 1412-1416.

Yan, H. and Chao, M. V. (1991) Disruption of cysteine-rich repeats of the p75 nerve growth factor receptor leads to loss of ligand binding. J. Biol. Chem., 266, 12099-12104.

Yan, Q., Matheson, C. and Lopez, O. T. (1995) In vivo neurotrophic effects of GDNF on neonatal and adult facial motor neurons. Nature, 373, 341-344.

Zarling, J. M., Shoyab, M., Marquardt, H., Hanson, M. B., Lioubin, M. N. and Todaro, G. J. (1986) Oncostatin M: a growth regulator produced by differentiated histiocytic lymphoma cells. Proc. Natl. Acad. Sci. USA, 83, 9739-9743.

Zurn, A. D., Baetge, E. E., Hammang, J. P., Tan, S. A. and Aebischer, P. (1994) Glial cell line-derived neurotrophic factor (GDNF), a new neurotrophic factor for motoneurons. Neuroreport, 6, 113-118.

Zurn, A. D., Winkel, L., Menoud, A., Djabali, K. and Aebischer, P. (1996) Combined effects of GDNF, BDNF, and CNTF on motoneuron differentiation in vitro. J. Neurosci. Res., 44, 133-141.